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13. ABSTRACT (Maximum 200 Words)
Primate and mouse display marked morphological differences upon their entrance to gastrulation. The most overt of these are topological dissimilarities of the gross embryos. While primate embryos develop as a planar epiblast within a spheroidal cytotrophoblastic shell, mouse epiblast develops in a cup shape, surrounded principally by a cylindrical endoderm shell. At this stage, the bulk of the murine trophoblast is limited to the mesometrial pole of the cylinder, rather than encapsulated by the cytotrophoblast as in the primate embryo. Additionally, the appearance of the primate mesoderm is precocious with respect to that of the mouse. Primate extraembryonic mesoderm is evident in sectioned tissues prior to the established of a primitive streak or node. In the mouse, such mesoderm appears only after establishment of these structures.
We are developing a new model of non-human primate biology using interspecific chimeras produced within tetraploid mouse blastocysts. By using tetraploid blastocysts in…

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INTRODUCTION

The relationship of extraembryonic membrane function to the progression of cancers, specifically cancers of the breast, is an understudied area of research. By understanding the factors, which control the molecular architecture of the placenta and other fetal membranes, we hope to shed light on mechanisms by which placental function is established. The initial goal of this research had been to characterize the role of the gene *mPlab* in mice conditionally null for the gene. *mPlab* is a member of the TGF-β superfamily of transforming growth factors, and had been previously demonstrated to be expressed at highest levels in the developing placenta. Midway through our research, we discovered that another laboratory had published the phenotype of the *mPlab* deficient mice (1). In light of this, we were forced to abandon our research on *mPlab* and find other areas of fetal membrane research to explore. In this report, we detail the completed work on our revised proposal. Additionally, the DOD had indicated that it would not be willing to fund research requiring work with non-human primate materials as described in the annual report dated June 2001. In order to alleviate these concerns, we submitted a modification of the new research proposal to the reviewing boards describing the development of a new technology modeling the early embryology of primates by the use of primate and rat ES cells in combination with mouse embryos.

BODY.

The goal of our original project was to characterize the loss of function of MPLAB in transgenic mice. In the May 2000 issue of Molecular and Cellular Biology, Se-Jin Lee's laboratory at Johns Hopkins University School of Medicine reported successful generation of a null allele of this gene and the resultant phenotype (1). With regards to the Statement of Work supplied with our original DOD funding application, we had successfully completed approximately half of the work contracted with the Department of Defense. We had independently cloned and determined the sequence of *mPlab*(*gdf-15*), constructed a targeting vector to generate a conditionally null allele of the gene, and generated transgenic embryonic stem cells from that vector.

Our research over the intervening time shifted to the development of an entirely new set of specific aims which seeks to test the mouse model as a legitimate model for primate embryology, especially with regard to the early establishment of the extra-embryonic membranes. Extraembryonic membrane biology was also the focus of the *mPlab* (*gdf-15*) study.

The basic premise of this research is that we, as a mouse research laboratory, use the mouse as a model to study early embryonic development for the ultimate purpose of understanding development in other mammalian species, specifically the human. The natural history of the mouse, as well as the availability of multiple congenic lineages, have made it particularly attractive as a genetic tool for research in numerous biological phenomena. The underlying assumption is, however, that the mouse closely models the biology of other animal systems. The reality of the matter is that the laboratory mouse represents only 1 of roughly 5000 mammalian species. Molecularly, there are numerous documented cases where research in the mouse or human does not faithfully predict the biology of the reciprocal species (2).

This is probably most carefully studied in pharmacokinetic literature, but is increasingly evident in other fields. A review of recent literature reveals mouse-human differences in the biologies of menopause (3), breast cancer (4,5), ovarian function (6), and wound repair (7) among others (8,9,10).

In my specific interest of developmental biology, it is known for example that many of the functions carried out by human placental estrogens, are performed in mice by a wholly dissimilar class of molecules, the prolactins. The mouse placenta is, in fact, incapable of producing

aromatase, an enzyme necessary for production of estrogens. Promoter studies of the human aromatase gene, however, have shown that elements of the human aromatase promoter can drive reporter gene expression in the murine placenta (11). This suggests that although the mature organ biology is quite different, the underlying molecular architecture of the two species may still be quite conserved. There exists, therefore, a class of placental research for which the tools available to murine biologists may provide significant advances in understanding of primate placental development and function. For these same reasons, however, it is necessary for mouse researchers to test the fidelity of their models in other species in order to assess where the limitations of the mouse may lie in any given field of research.

This project centered on the observation that primate and murine gastrulae, as well as those of other mammalian orders, display markedly disparate morphologies. This, of course, leads to the question of whether the differing morphologies are of similar molecular determination, or whether they are uniquely derived with respect to one another. A survey of comparative early embryology. "Eakin and Behinger 2004 "Germ layer formation in other mammals" is attached as a pre-print.

We are currently generating a new interspecific chimera technology to establish and model primate (or other xenotypic mammalian embryos) within the mouse, using tetraploid mouse cells to substitute early placental functions of the xenotypic embryo. We are, in essence, asking the question: "Can the extraembryonic tissues of the mouse support the early development of xenotypic embryos?" This technology takes advantage of previously established embryonic stem cell lines of the Cynomologus macaque (12) and did not require the procurement of any primates. Concurrently, interspecific chimeras of the rat and mouse were also produced. The rat:mouse studies are based on a series of papers in published in the 1970's in which successful embryonic chimeras of rat and mouse embryos were produced. These chimeras were generally created by either morula aggregation or by injection of rat inner cell masses into mouse blastulae. Using species specific antibodies, it was seen that the rat cells are capable of colonizing epiblast and trophectoderm tissues, but were twice as abundant in the endodermal lineages (13). In another study, which included 7.5 day embryos, rat contribution to mesoderm was seen (14). Though one paper reported the ability to culture these chimeras to 9.5 days of development (13), it was later found that a strong selection against rat cells had occurred and that these embryos consisted of little or no rat tissue (15). Such negative selection was blamed on maternal immune response, and was also seen in more closely related interspecific chimeras. In *M. caroli:M. musculus* ICM injection chimeras, this negative selection could be overcome by ensuring that the recipient blastocyst was of the same strain as the host mother (16). This suggests that trophoblast tissue is responsible for the protection of the interspecific tissue. The alternative explanation is that a heterogeneous trophoblast simply does not function at a level required for maintaining the life of the conceptus. Despite the problems, these papers proved that rat and mouse chimeras were capable of developing in concert to produce viable gastrulae.

Today, tetraploid embryos can be used in combination with embryonic stem cells to produce embryos of 100% ES cell derivation inside a trophoblast shell of 100% host embryo derivation (17). We have been addressing the question, "Can the trophoblast tissue of a mouse support to early development of a xenotypic embryo?". Our hypothesis is that a tetraploid mouse trophoblast will be able to shield the developing xenotypic embryo from the maternal immune system and provide a suitable surrogate placenta for the early development of the xenotypic embryo.

While reviewing pertinent literature on tetraploidy in mice it became apparent that studies initially describing embryonic stem cell growth in tetraploid blastocysts had only been performed on embryos older than E8.5. Since gastrulation occurs prior to E8.5 it is essential to describe 4N:ES cell chimera development at earlier stages to insure that the distributions of tetraploid and ES cells are truly segregated as described in later development. Furthermore, it became apparent that analysis of previous studies was complicated by a lack of consistency in the genetic background of the mice in which tetraploidy was induced. For more complete description please refer to Eakin and Behringer (2003) "Tetraploid Development in the Mouse" (attached).

In order to address the contribution of genetic background to the developmental potential of tetraploid mice we began by analyzing tetraploid embryos produced by electrofusion of 2-cell staged embryos. Tetraploid embryos of Swiss Webster and CBA Bl6 F2 strains were observed develop in one of four general manners. Many embryos either developed as a cluster of trophoblast giant cells (Fig 1a), or arrested prior to gastrulation (Fig 1b). Embryos that escaped early lethality either developed as extensive outgrowths of extraembryonic tissue (fig 1d). Although these have been called "empty chorionic vesicles", due to their apparent lack of embryonic tissue, histological sections revealed limited amounts of embryonic ectodermal structures (fig 1c). Rarely, embryos of either strain were observed to develop advanced embryonic structures. In these cases 4n CBABl6 F2 (fig 1e). A totaled summary of the recovery of the transferred embryos is presented in table I.

In order to assess the degree to which genetic background effects developmental potential, we examined tetraploid embryos at time points through the first half of gestation. 4n CBABl6 F2 transfers consistently resulted in the return of 4n conceptuses more rarely than transfers of 4n Swiss embryos (Figure 2). Outgrowths of similar to "empty chorionic vesicles" (fig 1c,d) were most commonly recovered. At days 9.5 and 10.5, advanced embryonic development (fig 1e) was observed three times in Swiss background (n=132 transferred embryos), as compared to once in the 4n CBABL6 F2 background (n=109 transferred embryos). Control transfers of 2n Swiss embryos were recovered at 76% on day 10.5. As tetraploid embryos develop more slowly than diploid controls, the lower recoveries seen at early time points may reflect the difficulty involved in dissecting pre-gastrulation staged embryos.

In order to address whether the reduced viability of 4n CBABl6 F2 embryos was manifested prior to embryo transfer we observed the pre-implantation development of tetraploid embryos of either strain during *in vitro* embryo culture. After induction of tetraploidy 96% (n=116) of 4n Swiss embryos developed to the blastocyst stage within 72 hours, compared with 86% (n=33) of 4n CBABl6 F2
embryos. Swiss diploid controls developed from 2 cell stage to blastocyst at 100% efficiency (n=30), compared with 2n CBABl6 F2 controls which developed at 91% (n=36) in our culture conditions.

In an effort to determine whether the timing of the cell cycle in the two strains is perturbed, we initiated a pilot study to observe the rate of cell division during *iv vitro* culture of cleavage stage embryos. This was done by inferring rates of division from blastomere counts taken at 6 hour intervals (fig 3). Differences between the rates of cell division could not be satisfactorily determined from this experiment. In the future, this experiment will be repeated using vital fluorescent dyes in conjunction with time lapse photography. Additionally, whole mount staining of KI-67 and Annexin 5 (Molecular Probes) will be used to quantify the number of apoptotic and mitotic cells in 4n embryos.

In wild-type diploid embryos, the paternal X chromosome is selectively inactivated in the extraembryonic tissue and randomly inactivated in embryonic regions (Fig. 4a). In order to determine whether imprinting of the sex chromosomes is perturbed in tetraploid embryos we induced tetraploidy in embryos produced from the mating of male Swiss mice bearing an X-linked cytoplasmic GFP transgene to wild-type Swiss female mice. Female embryos were then scored for distribution of GFP expression. Only two female GFP+ embryos were found (Fig. 4b). In these, the paternal X chromosome appears to have been normally imprinted in the extraembryonic tissue, but may also be down regulated in the embryonic tissue relative to diploid controls. These experiments will be repeated to get larger sample sizes.

In order to test whether tetraploid cells were capable of contributing to embryonic lineages in gastrulation stage 2n:4n chimeras, we injected diploid eGFP tagged embryonic stem cells into tetraploid mouse blastocysts. After transfer to pseudopregnant foster mothers, the embryos were harvested at e6.5 and counterstained with 10 uM Draq5 nuclear stain (Biostatus, Ltd). The cells of the epiblast were then scored for GFP expression. Cells that did not express GFP were presumed to be tetraploid. The genotype of the tetraploid blastocyst was either Swiss Webster or CBABl6 F2. Out of 126 injected blastocysts, 11 GFP+ chimeras were harvested at e6.5 (Table 2). Preliminary evidence suggests that tetraploid cells do contribute to the epiblast at early stages (Fig. 5). Additionally, it appears that tetraploid cells of the Swiss Webster background may contribute to epiblast tissues more frequently than tetraploid cells of the CBABl6 F2 background. Conclusions regarding the latter observation await repetition of the experiment to obtain larger sample sizes.

Using tetraploid complementation, we have been producing 4n mouse:2n rat and 4n mouse:2n macaque interspecific chimeras. To date, these studies have resulted only in low percentage chimerism.

Rat embryonic cells were derived disaggregation of Inner Cell Mass (ICM) outgrowths. In the first experiments ICM outgrowths were cultured with FM4-60, a vital dye (Molecular Probes). Dissaggregated inner cell mass outgrowth were combined with tetraploid 2 cell mouse embryos carrying a cytoplasmic GFP transgene. In these studies it was hoped that the fate of rat cells could be followed in short term culture by retention of the FM4-60 dye. In practice, FM4-60 loaded rat cells were not taken up into the tetraploid mouse embryo, and instead remained attached to the periphery of the chimera.

Since it was possible that growth and division of the rat cells was causing the FM4-60 to be diluted below the limits of detection, we repeated the experiment using rats expressing a ubiquitous GFP transgene (obtained from the National Rat Resource and Research Center). Although we were able to document GFP+ expression in the ICM of chimeric blastocysts (Fig 7), the rat cells were never observed after transfer into pseudopregnant host mice, except in one case. In that instance, GFP+ rat cells were observed in the trophoblast of the chimeric egg cylinder (Fig. 8), suggesting that the ICM outgrowth protocol was likely resulting in rat trophoblast contamination.

Future experiments, will involve isolation of rat ICM by immunosurgery, and injection of rat cells into the tetraploid mouse blastocyst rather than aggregation.

Interspecific chimeras of the macaque and mouse have been documented at the pre-implantation stage (Fig. 9a). In these aggregation chimeras GFP+ macaque ES cells (11) were combined with 4n 2 cell mouse embryos. After transfer to pseudopregnant host mice, GFP

expression was never observed. In a separate experiment, single macaque ES cells were injected into 4n mouse blastocysts and cultured overnight. In these chimeras, the ES cells were not observed to divide after 24 hours of culture (Fig 9b). The low return of these experiments is likely due to technical problems thawing culturing macaque ES cells in our laboratory. We are currently negotiating the shipment of live, rather than frozen, cell cultures from our collaborator and plan on injecting 4n mouse blastocysts with cells derived from these cultures.

KEY RESEARCH ACCOMPLISHMENTS

- Cloning and sequencing of the *mPlab* gene
- Creation of a targeting vector for the production of a conditionally null allele of *mPlab*.
- Establishment of an ES cell lines putatively heterozygous for the *mPlab* conditionally null allele.
- Establishment of tetraploid production, culture, and aggregation chimera technologies and in our laboratory
- Documentation of differing developmental potentials between tetraploid mouse embryos of different genetic backgrounds.
- Documentation of the presence of tetraploid cells in the embryonic regions of egg cylinder staged tetraploid:diploid chimeras.
- Production of "low percentage" interspecific chimeras of 2n mouse:2n rat, 4n mouse:2n rat, and 4n mouse:2n Cynomologus macaque.

REPORTABLE OUTCOMES

Reportable outcomes for DAMD 17-1-0311, as described in the "training reporting requirements" http://mrmc-www.army.mil/rrptraining.asp.

1. Eakin, GS and Behringer, RR. (2003) Tetraploid Development in the Mouse. *Developmental Dynamics,* 228(4):751-766.
2. Eakin GS and Behringer RR. (in press, due April 2004). Germ Layer Formation in Other Mammals. In *Gastrulation: From Cells to Embryos* (ed. Stern C). Cold Spring Harbor Press. Cold Spring Harbor, New York.

Abstracts

1. Eakin, GS and Behringer, RR. "Molecular Embryology of the Non-human Primate." Department of Defense Breast Cancer Research Program Era of Hope Meeting. September 2002, Orlando, Florida.
2. Eakin, GS, Nakatsuji, N, and Behringer, RR. "Generation of Primate:Mouse Interspecific Chimeras." Society for Developmental Biology 62nd Annual Meeting. August 2003, Boston Massachusetts.

CONCLUSIONS

An unfortunate consequence of the large scale and fast-pace of scientific research is that often multiple laboratories are working on closely related projects, and may be unaware of the other's efforts. This was the case with our research on the *mPlab* gene. In late December, 2000, we became aware that our effort to construct null alleles of the murine *mPlab* gene was a

duplication of published work in another laboratory. It was apparent that continued research on mPlab would not be a productive endeavor for either the laboratory or the education of the graduate student (G.E.) performing the research. As such we designed a new project, submitted in the previous annual report, outlining a research program intended to test the limitations of the mouse system as a model for primate early development and disease. In this annual review we address concerns of the reviewing board and propose an alternative technology for studying primate embryonic development using only pre-existing non-human primate embryonic stem cells. This research is of crucial step in the establishment of mouse models as numerous very basic differences between the biologies of mice and primates are appearing in the literature. Cancer is diagnosed during 1 in 1000 pregnancies. As more and more women delay their first pregnancies into later years, the incidence of cancer diagnosis during pregnancy is expected to increase. Our ability to bring the power of mouse research to bear against these questions rests, therefore, on our knowledge of the limitations to which the mouse can serve as a model for these diseases.

The distribution of tetraploid cells in 4n:2n chimeras has not been reported at gastrulation, though it has, in practice, been assumed to mirror the discrete compartmentalization seen in later stages. Additionally, although an influence of genetic background on the developmental potential of tetraploid embryos has been hypothesized it has not been rigorously documented.

We have documented a genetic background effect on the developmental potential of tetraploid embryos. In 4n embryos transferred to pseudopregnant foster mothers, embryos from the Swiss-Webster background displayed more frequent and more advanced development of embryonic structures than did embryos of a CBAB16 F2 background. As Swiss Webster are out-bred, and the CBAB16 F2 mice are hybrids of two inbred strains, genetic mechanism of the disparities in developmental potential is most likely due to the greater gene pool available to developing Swiss-embryos. Additionally, we have observed significant contribution of tetraploid cells to the epiblast of gastrulation staged mouse embryos. The influence of genetic background in these chimeras is suggested, but requires further controls and repetition. The significance of these results to other researchers is that they call into question the conclusions of several previous publications that used tetraploid embryo complementation to segregate extraembryonic and embryonic lineages at gastrulation. These results additionally suggest the inclusion of additional genetic controls in 4n:2n chimera studies.

The production of interspecific chimeras of the mouse, rat and macaque is an ongoing endeavor. To date, xenotypic species contribution to the 4n host mouse embryo has been limited mainly to pre-implantation development. Technical refinements of the chimera-making protocols are currently being pursued.

REFERENCES

1. E.C. Hsiao, et al. Characterization of growth-differentiation factor 15, a transforming growth factor beta superfamily member induced following liver injury. *Mol Cell Biol* **20** 3742-51 (2000).
2. A. Wynshaw-Boris. Model mice and human disease. *Nat Genet* **13**, 259-260 (1996).
3. F.L. Bellino. Nonprimate animal models of menopause: workshop report. *Menopause* **7**, 14-24 (2000).
4. A.D. Cardiff. Validity of mouse mammary tumour models for human breast cancer: comparative pathology. *Microsc Res Tech* **52**, 224-230 (2001).
5. S. Nandi. Hormones and mammary carcinogenesis in mice, rats, and humans: a unifying hypothesis. *Proc Natl Acad Sci U S A.* **92**, 3650-3657 (1995).
6. B.S. Dunbar et al. The ovary as an immune target. *J Soc Gynecol Investig* 8 (1 Suppl Proceedings):S43-8 2001
7. A.B. Borisov. Regeneration of skeletal and cardiac muscle in mammals: do nonprimate models resemble human pathology? *Wound Repair Regen* **7**, 26-35. (1999).
8. C.M. Disteche. Escape from X inactivation in human and mouse. *Trends Genet* **11**, 17-22 (1995).
9. W.E. Wright and J.W. Shay. Telomere dynamics in cancer progression and prevention: fundamental differences in human and mouse telomere biology. *Nat Med* **6**, 849-851 (2000).
10. S.V. Prasad et al. Structure and function of the proteins of the mammalian Zona pellucida. *Cells Tissues Organs* **166**, 148-164 (2000).
11. A. Kamat, K. H. Graves, M. E. Smith, J. A. Richardson, C. R. Mendelson. A 500-bp region, approximately 40 kb upstream of the human CYP19.*Proc Natl Acad Sci U S A* **96**, 4575-80 (1999).
12. Suemori H, Tada T, Torii R, Hosoi Y, Kobayashi K, Imahie H, Kondo Y, Iritani A, Nakatsuji N. (2001). *Dev Dyn.* **222** :273-279.
13. Gardner, R. L. and M. H. Johnson (1973). "Investigation of Early Mammalian Development using Interspecific Chimeras between Rat and mouse." Nature New Biology **246**(151): 86-89.
14. Rossant, J. (1976). "Investigation of inner cell mass determination by aggregation of isolated rat inner cell masses with mouse morulae." J Embryol Exp Morphol **36**(1): 163-74.
15. Gardner, R. L. and M. H. Johnson (1975). "Investigation of cellular interaction and deployment in the early mammalian embryo using interspecific chimaeras between the rat and mouse." Ciba Found Symp **0**(29): 183-200.

16. Rossant, J., B. A. Croy, et al. (1982). "Interspecific chimeras in mammals: a new experimental system." J Anim Sci **55**(5): 1241-8.
17. Nagy, A., E. Gocza, et al. (1990). "Embryonic stem cells alone are able to support fetal development in the mouse." Development **110**(3): 815-21.

Figure 1.

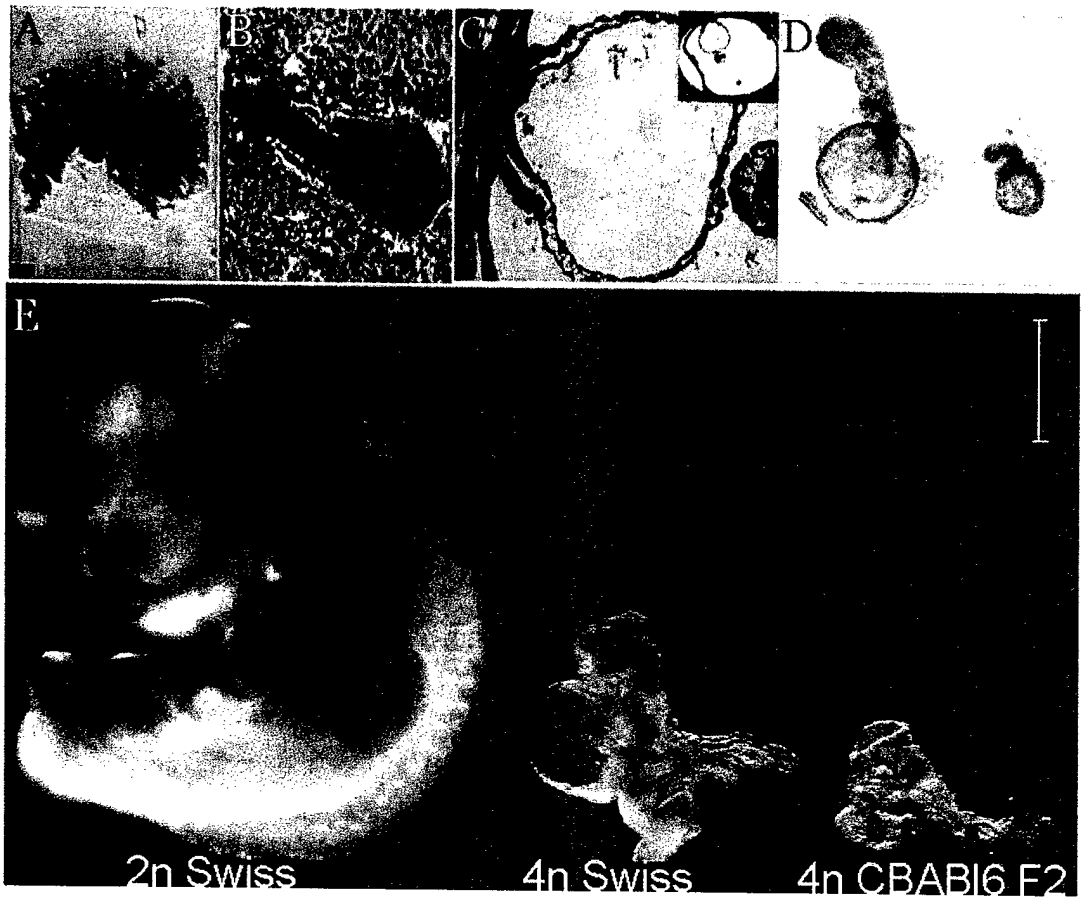


Table I Recovery of Tetraploid Embryos Transferred to Pseudopregnant Females .

	Embryos transferred	Decidual Reactions	Recovered 4n embryos
4n Swiss	289	130	67
4n CBABl6F2	201	73	12
Total	490	203	79

Figure 2.

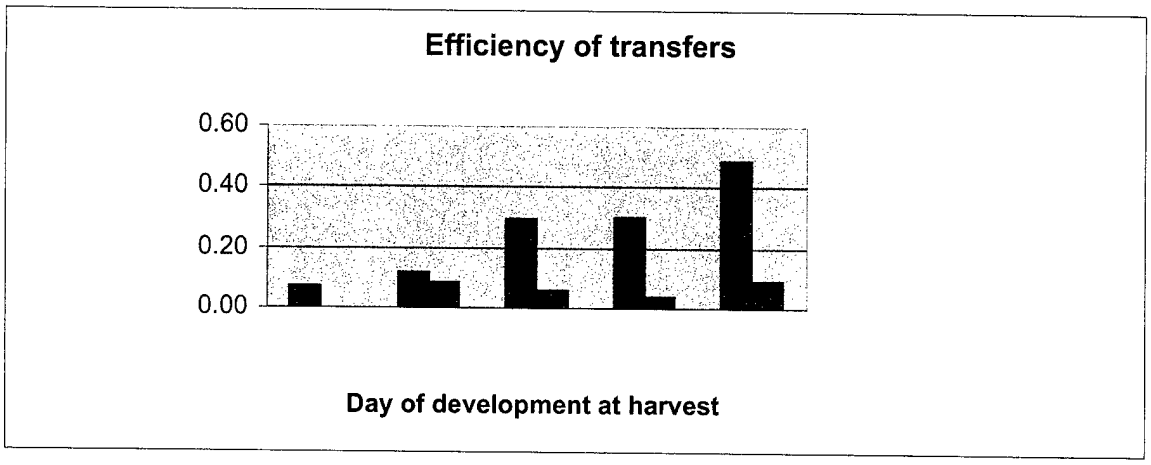

Efficiency of transfers
0.60
0.40
0.20
0.00
Day of development at harvest

Figure 3

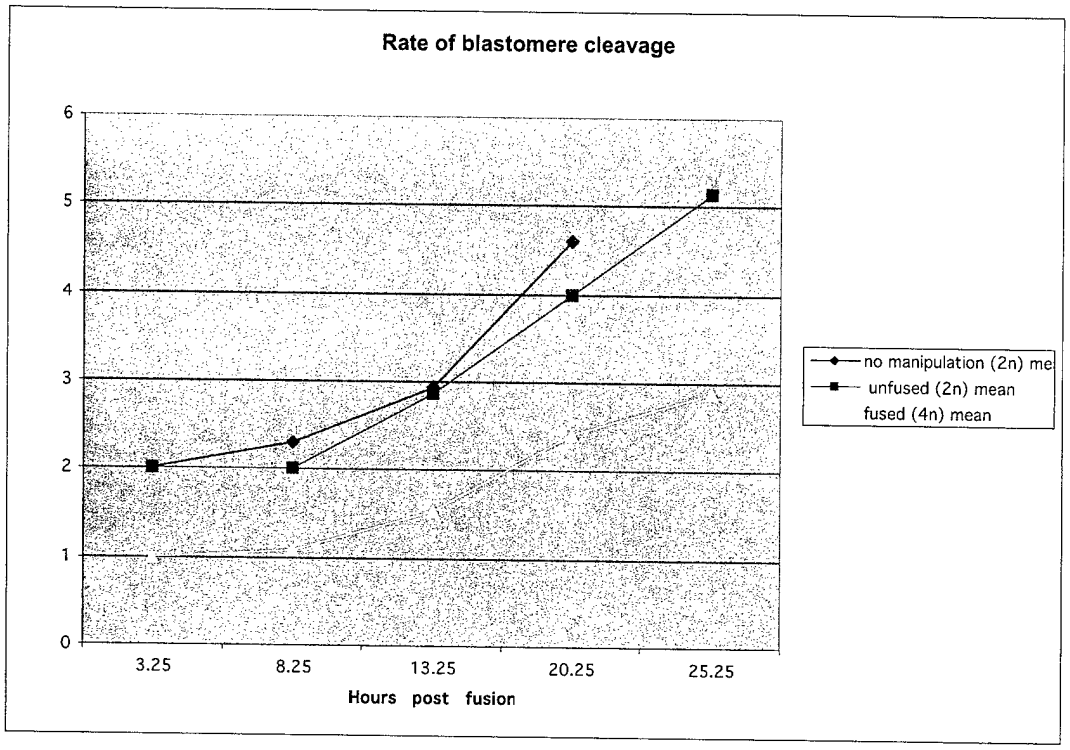

Rate of blastomere cleavage
6
5
4
3
2
1
0
3.25
8.25
13.25
20.25
25.25
Hours post fusion
no manipulation (2n) me
unfused (2n) mean
fused (4n) mean

Figure 4

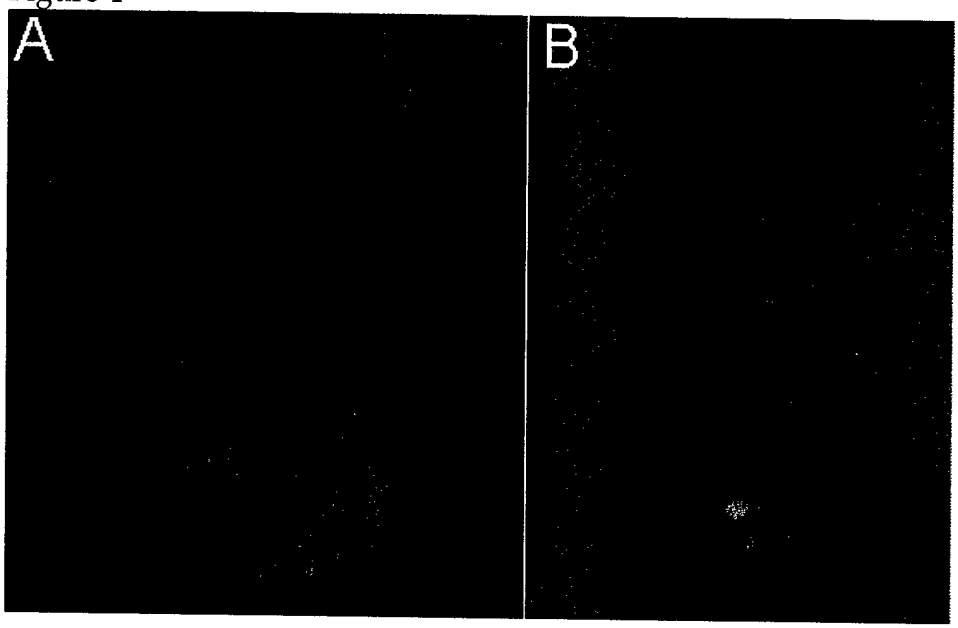
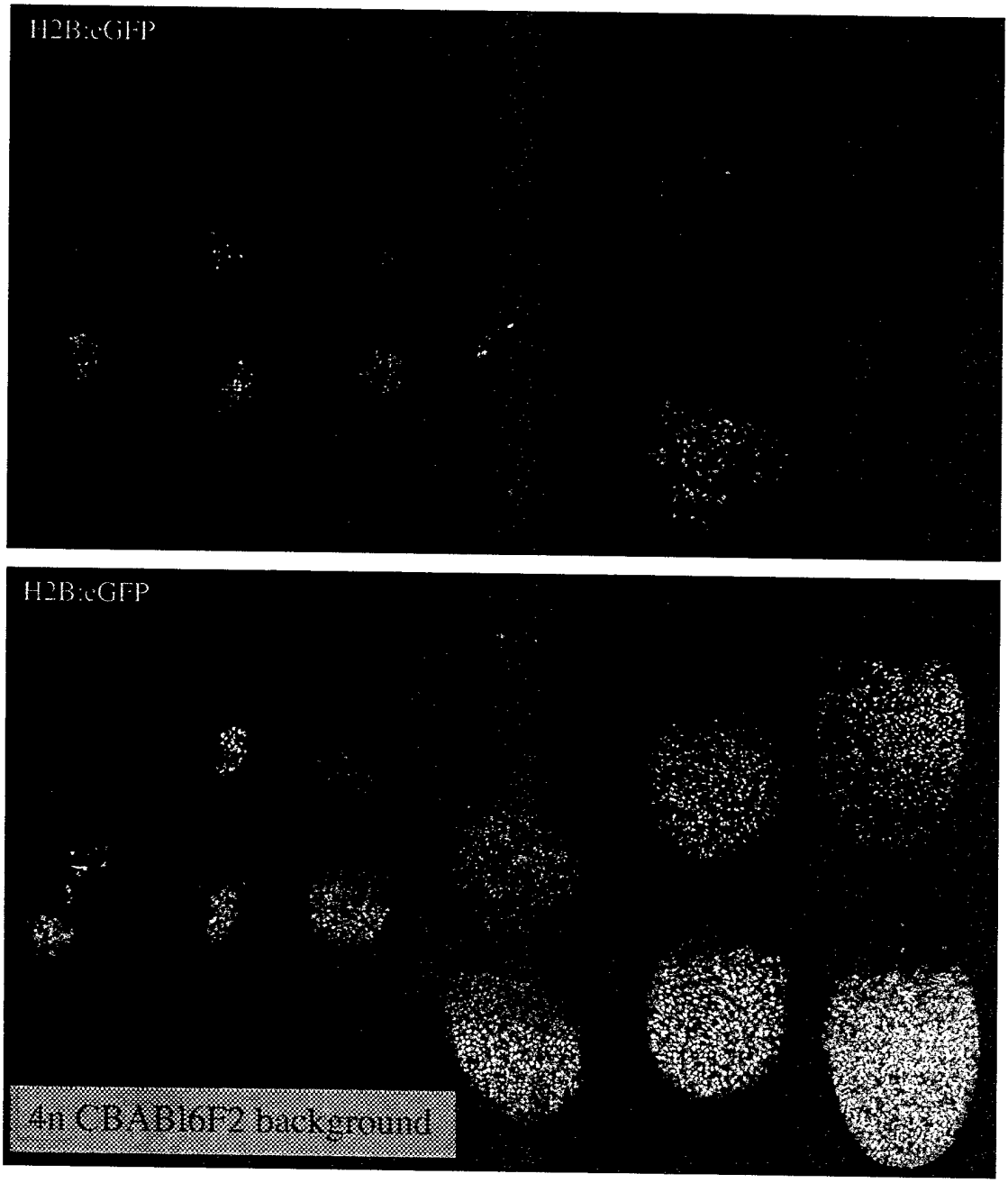


Table 2

Strain	Embryos injected	Early gestation embryos recovered	GFP+ chimeras	GFP+/ Recovered	GFP+/ Injected
2n Swiss	17.00	11.00	8.00	72.73	47.06
4n Swiss	80.00	21.00	5.00	23.81	6.25
4n CBAB6F2	46.00	9.00	6.00	66.67	13.04

Figure 5



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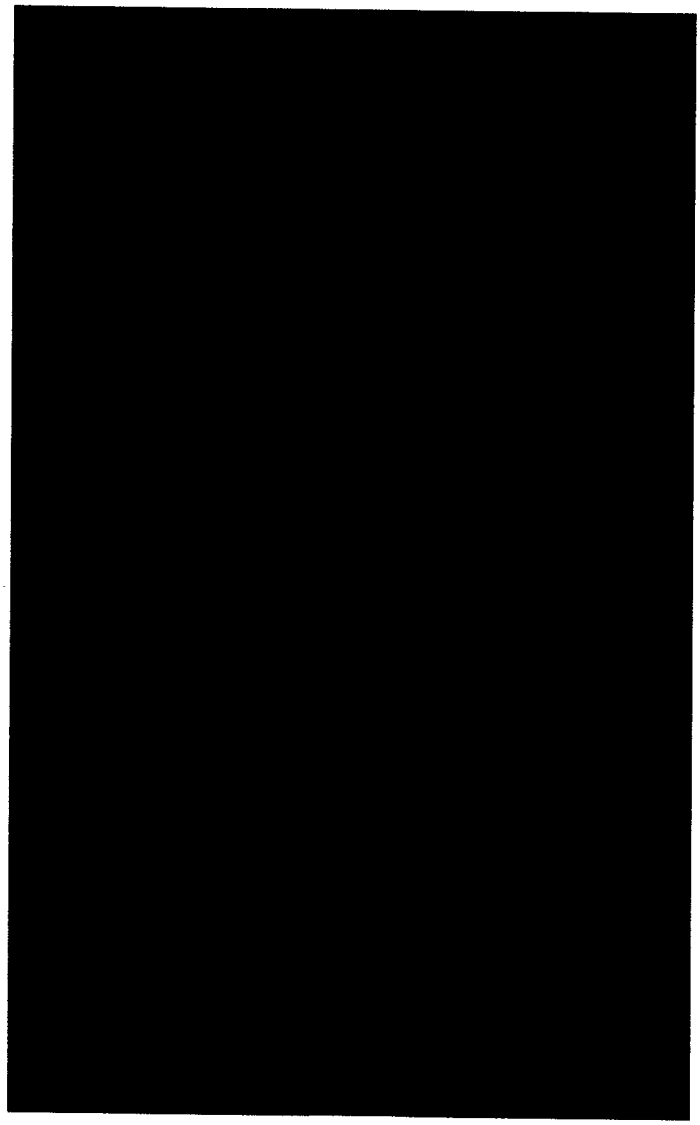


Figure 7

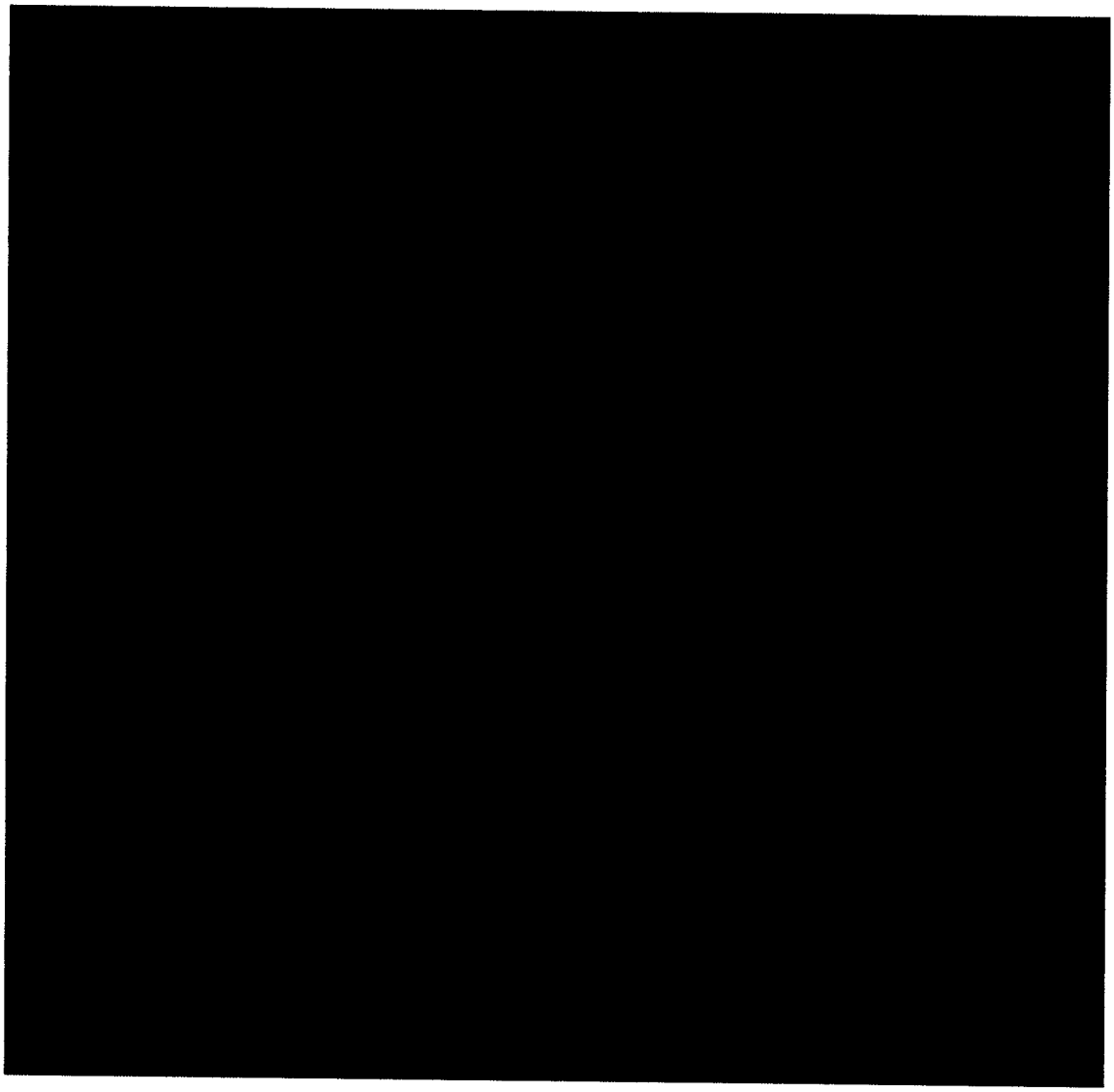


Figure 8

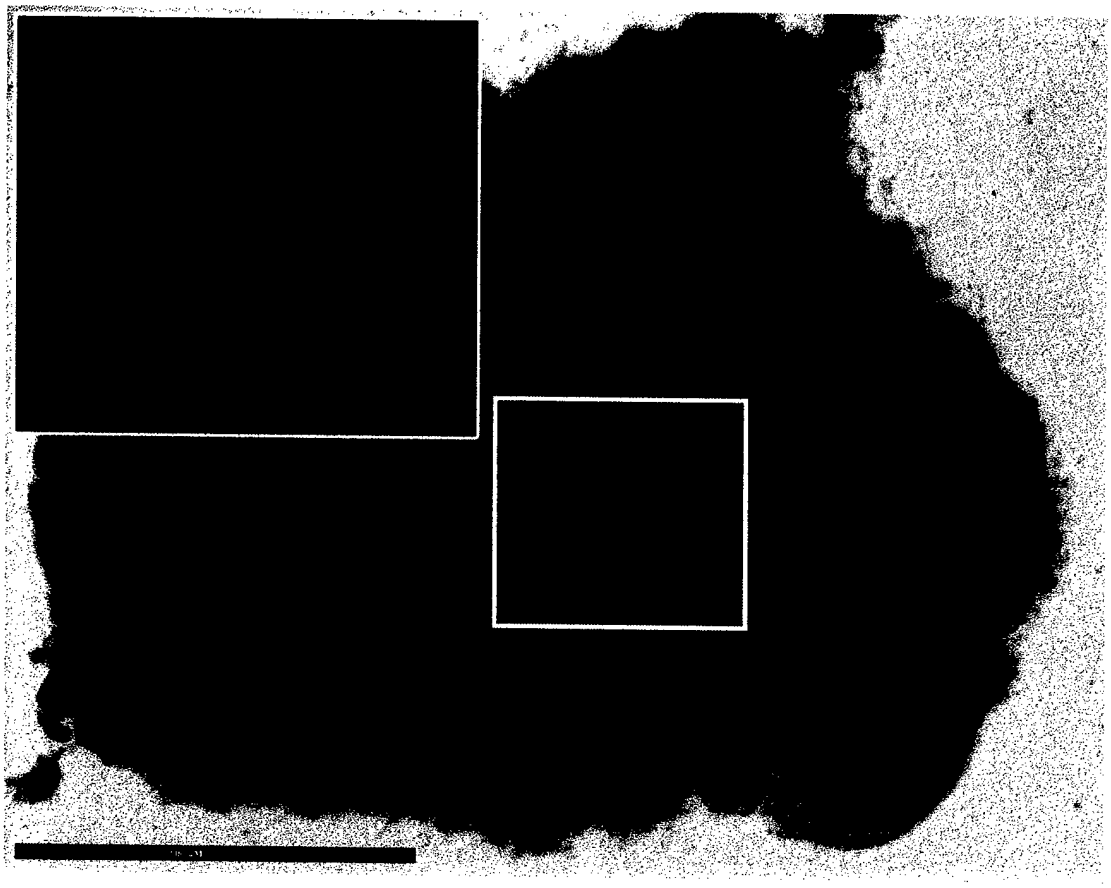
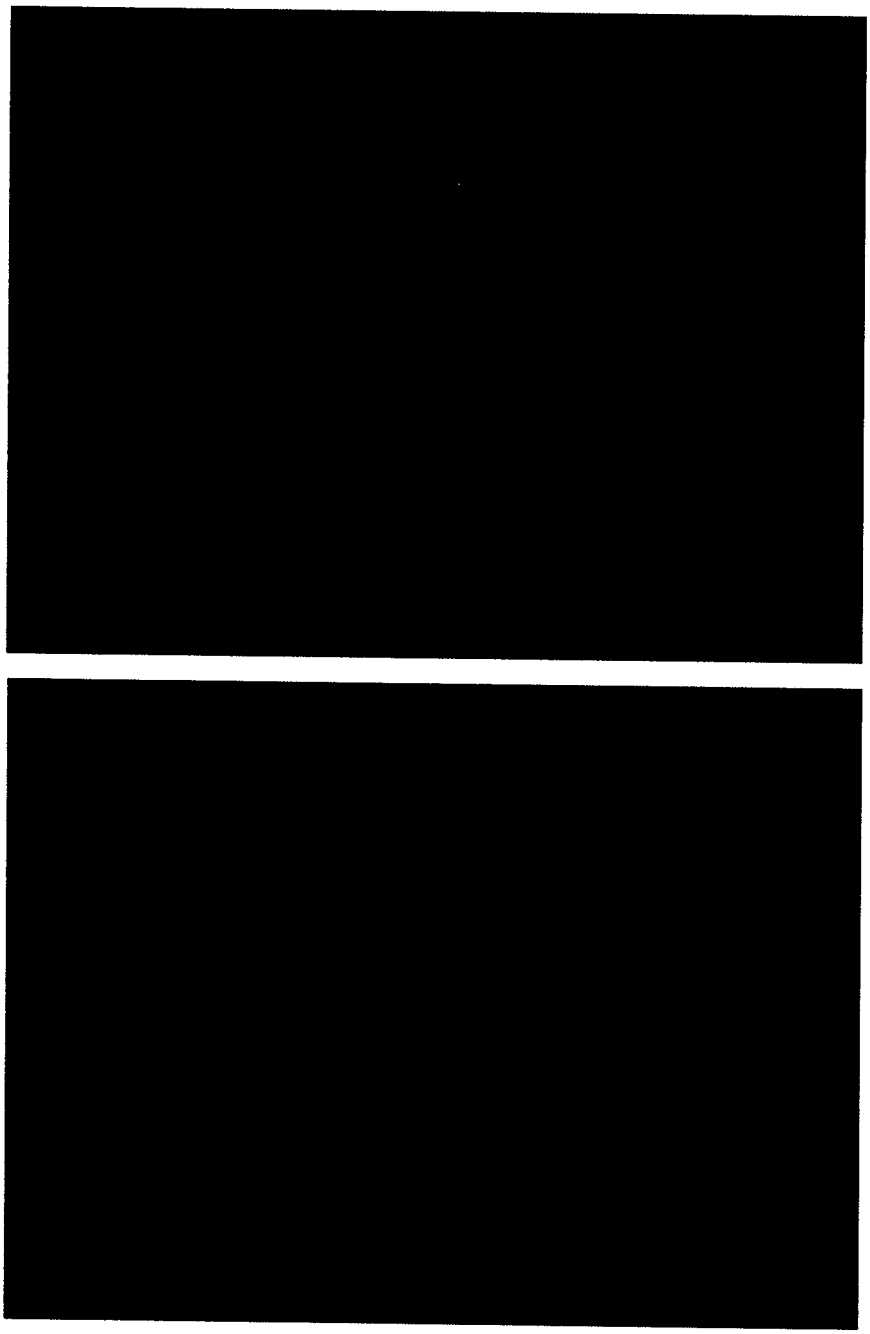


Figure 9



APPENDICES

BIBLIOGRAPHY

PUBLICATIONS AND MEETING ABSTRACTS EMANATING FROM DAMD 17-1-0311

Functional characterization of the murine homologue of PLAB, a novel TGF-B member, in placental and mammary tissues. (Reprinted from "Reportable Outcomes")

Publications

1. Eakin, GS and Behringer, RR. (2003) Tetraploid Development in the Mouse. *Developmental Dynamics,* 228(4):751-766.
2. Eakin GS and Behringer RR. (in press, due April 2004). Germ Layer Formation in Other Mammals. In *Gastrulation: From Cells to Embryos* (ed. Stern C). Cold Spring Harbor Press. Cold Spring Harbor, New York.

Meeting Abstracts

1. Eakin, GS and Behringer, RR. "Molecular Embryology of the Non-human Primate." Department of Defense Breast Cancer Research Program Era of Hope Meeting. September 2002, Orlando, Florida.
2. Eakin, GS, Nakatsuji, N, and Behringer, RR. "Generation of Primate:Mouse Interspecific Chimeras." Society for Developmental Biology 62nd Annual Meeting. August 2003, Boston Massachusetts.

Personnel receiving pay from DAMD-17-1-0311

Guy S Eakin.

ATTACHED REPRINTS.

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DEVELOPMENTAL DYNAMICS 228:751–766, 2003

Tetraploid Development in the Mouse

Guy S. Eakin and Richard R. Behringer*

Spontaneous duplication of the mammalian genome occurs in approximately 1% of fertilizations. Although one or more whole genome duplications are believed to have influenced vertebrate evolution, polyploidy of contemporary mammals is generally incompatible with normal development and function of all but a few tissues. The production of tetraploid (4n) embryos has become a common experimental manipulation in the mouse. Although development of tetraploid mice has generally not been observed beyond midgestation, tetraploid:diploid (4n:2n) chimeras are widely used as a method for rescuing extraembryonic defects. The tolerance of tissues to polyploidy appears to be dependent on genetic background. Indeed, the recent discovery of a naturally tetraploid rodent species suggests that, in rare genetic backgrounds, mammalian genome duplications may be compatible with the development of viable and fertile adults. Thus, the range of developmental potentials of tetraploid embryos remains in large part unexplored. Here, we review the biological consequences and experimental utility of tetraploid mammals, in particular the mouse. ***Developmental Dynamics 228:751–766, 2003.*** **© 2003 Wiley-Liss, Inc.**

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INTRODUCTION

In vertebrates, each chromosome of most somatic cells is paired with one sister somatic chromosome or with a sex chromosome to form a diploid genome. As a result of cell fusion or mistakes during cell division, cells and occasionally whole organisms can become polyploid wherein the ploidy of the genome accumulates several multiples of the haploid genome number (n).

Some tissues (e.g., skeletal muscle, hepatocytes, megakaryocytes, urinary bladder epithelia, myocardium, syncytiotrophoblast, and corpora lutea) are often polyploid due to endoduplication of the genome or cell fusion during normal development (Keighren and West, 1993). Other cells, such as the trophoblast giant cells, are considered to be polytene, having greater numbers of chromatids per chromosome, rather than polyploid, having greater numbers of segregating chromosomes. Having multiple copies of the genome confers certain advantages to cells such as resistance to effects of genome injury. Additionally, increases in cell size, due to polyploidy or polyteny, permit greater flexibility and strength in tissues that are subject to mechanical stresses, such as the urinary bladder epithelia (Brodsky and Uryvaeva, 1985). Greater cell size also allows for tissues to develop using fewer cells (Brodsky and Uryvaeva, 1985). The molecular mechanisms governing specific cell-type resistance to polyploidy remain unclear. Most often, divergence of ploidy from the diploid (2n) norm results in a disease state.

Although tetraploidy in humans is rare (see below), the consequence of abnormal chromosome number and gene balance is a familiar event (Hassold and Hunt, 2001). The XO karyotype leads to Turner's syndrome, whereas most other monosomies produce inviable embryos. Trisomies of the sex chromosomes exist, with XXY (Klinefelter syndrome), XYY, and XXX appearing in roughly 1:1,000 births. Autosomal trisomies generally produce inviable embryos. Trisomies of chromosomes 8 and 9 cause lethal developmental defects; trisomies of human chromosomes 13, 18, and 21 (Down syndrome) are tolerated in so much as viable infants are born, although many do not survive the first few months of life. Those that do are mentally retarded and display mod-

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erate to severe developmental malformations (Larsen, 1993). The presence of congenital heart defects among individuals with Down syndrome has been correlated with three restriction length fragment polymorphisms in the region of *COL6A1* on chromosome 21 (Davies et al., 1995). This finding suggests that the severity of a syndrome caused by aneuploidy may be modified in part by the genetic background of the affected individual.

While mammals appear to be particularly sensitive to the detrimental effects of polyploidy, evolution has produced several organisms with varying values of n. Most familiar to developmental biologists are the amphibian *Xenopus laevis* and teleost *Danio rerio*. Both are considered to be evolutionary descendants of allotetraploid ancestors. Allotetraploid organisms are the consequence of the combination of two genomes of closely related species that contain genetically different chromosome sets (Reiger et al., 1991). Genome fusion is believed to have played a role in the recent evolution of only one mammal. The red viscacha rat, *Tympanoctomys barrerae*, possesses 100 autosomal chromosomes and two sex chromosomes, while its closest relatives possess only 55 autosomes and a single sex chromosome set (Contreras et al., 1990; Gallardo et al., 1999). In this case, it is notable that the apparent genome duplication includes neither the sex chromosomes nor a duplication of the autosomal 43rd chromosome pair, suggesting that these chromosomes may bear loci that are incompatible with polyploidy in vivo. It is not known whether cells that are normally polyploid in other species, such as the urinary bladder epithelia or trophoblast giant cells, exhibit proportional duplications of ploidy in *T. barrerae* (M. Gallardo, personal communication). Further studies of genomic organization and meiotic pairing will be required before it is known whether *T. barrerae* arose by allotetraploidy or by duplication of one ancestral genome (autotetraploidy). Such a distinction might provide hypotheses concerning whether resistance to polyploidy arose by "simple" modification of a duplicated single genome in the case of autotetraploidy, or whether resistance to tetraploidy required components unique to each of the two ancestral genomes.

Complete tetraploidy, as a general rule, is not compatible with normal development and viability. Rare instances of spontaneous tetraploidy are generally due to failure of cytokinesis at the first zygotic cell division, which, in mice, occurs at a frequency of approximately 0.1%. The incidence in rats, rabbits, and pigs of spontaneous tetraploidy is estimated at 0.4%, 0.3%, 0.1–3.4%, respectively (McFeely, 1969; reviewed in Dyban and Baranov, 1987), whereas the incidence of tetraploidy in bovine embryos produced in vitro is 2.8% (Kawarsky et al., 1996). Karyotypes of spontaneous human abortuses estimated the frequency of spontaneous tetraploidy to be between 1.1 and 7.1% (Carr, 1972; Creasy et al., 1976; Hassold et al., 1980; Kajii et al., 1980) with the majority falling in the lower range. These were usually characterized by empty chorionic sacs, lacking any embryonic tissue (Warburton et al., 1991). Although abortion generally occurs at a menstrual age of 11 weeks, it is assumed that embryonic death occurs significantly earlier (Carr, 1972). Despite the odds, nine complete tetraploid live births have been reported in the past 30 years. These infants displayed a myriad of defects, including spina bifida, skeletal and cartilaginous defects, as well as organ hypoplasia. Most also had facial dysmorphologies (Warburton et al., 1991).

EXPERIMENTAL PRODUCTION OF TETRAPLOIDY IN MAMMALS

As a research tool, polyploid embryos may provide useful insights into normal development. For reasons explained later, the polyploid embryo has been used to provide insight into the regulation of cell size, cell number, and rates of cell cleavage in early conceptuses. They have been used in lineage studies, as models for polysomic human conditions, and as a method for altering the balance of parental genomes. Most notably, tetraploid embryos are commonly used to rescue embryonic lethality as a result of defective extraembryonic phenotypes in laboratory mouse strains, as well as a method of generating mice directly from embryonic stem (ES) cells.

Because spontaneous embryonic tetraploidy is rare, tetraploid animals must be generated experimentally to determine the effect of polyploidy on growth and development. Various methods have experimentally induced tetraploidy in mammals. Three basic strategies are (1) to surgically add a diploid nucleus to a zygote, (2) to duplicate the genome without cell division using inhibitors of cytokinesis, or (3) to induce fusion of two diploid blastomeres (Fig. 1). The manufactured tetraploid animals can then be analyzed after culture in vitro or after transfer into recipient females and subsequent development. The following section describes several techniques used to induce tetraploidy; detailed accounts of phenotypes observed in these studies follow in later sections.

Nuclear Transfer Into Zygotes

The most direct route to tetraploidy is the physical injection of a donor diploid nucleus into a one-celled fertilized oocyte by using micromanipulators. Although originally performed in the rabbit (Bromhall, 1975), this technique was subsequently developed in the mouse as a means of testing developmental potencies of trophoblast and inner cell mass (ICM) cell nuclei after injection into blastocysts and subsequent development in vitro (Modlinski, 1978, 1981). Although this procedure may have produced the first repeatably uniform tetraploid embryos, the surgical trauma resulted in survival of only 9–15% of the injected blastocysts (Modlinski, 1981). Although the new method of piezo-actuated micromanipulation may increase the efficiency of nuclear transfer, the extensive equipment, skill, and time necessary for such manipulations makes tetraploid embryo production by microsurgery suitable only for

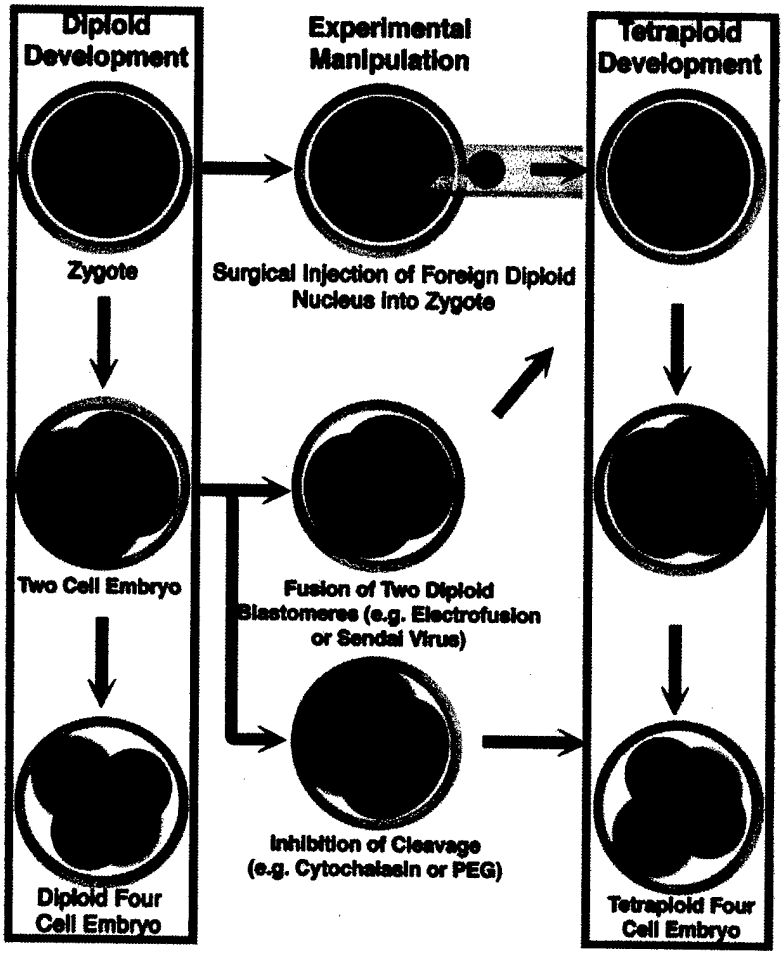

Fig. 1. Methods for production of tetraploid mouse embryos.

specialized nuclear transfer experiments.

Inhibition of Cleavage

A second method used to produce tetraploid embryos is to inhibit cleavage by chemicals or heat-shock. Initially, cytochalasin B (CB), a microtubule destabilizer, was used as the inhibitor (Snow, 1973; Tarkowski et al., 1977; reviewed in Niemierko and Opas, 1978). Because cytochalasins inhibit cytokinesis without disrupting karyokinesis, duplication of the genome was achieved. Inhibition of second cleavage (2 cell → 4 cell) was achieved by transient exposure of two-cell embryos with CB (Snow, 1973, 1975, 1976; Perry and Snow, 1975; Tarkowski et al., 1977; Niemierko and Opas, 1978; Lu and Markert, 1980; Kolzumi and Fukuta, 1995, 1996). While avoiding complications associated with embryo culture of one-cell embryos (Tarkowski, 1972), it is possible that the choice of two-cell embryos, rather than zygotes, may have produced several tetraploid:diploid (4n:2n) mosaics rather than homogeneously tetraploid animals. As many as 20% of embryos produced by CB may have been 4n:2n mosaics in these studies (Tarkowski et al., 1977). In this event, it is probable that, while one cell division was successfully inhibited, the

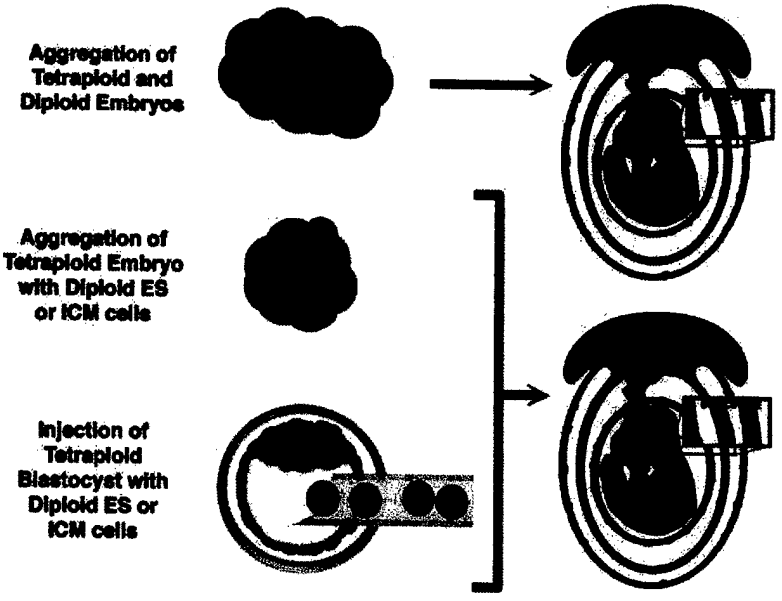

Fig. 3. Chimeras produced from tetraploid embryos. Red, diploid; green, tetraploid; purple, mixture of diploid and tetraploid cells. Magnification of embryo (A), amniotic ectoderm (B), amniotic mesoderm (C), yolk sac mesoderm (D), and yolk sac endoderm (E). ICM, inner cell mass.

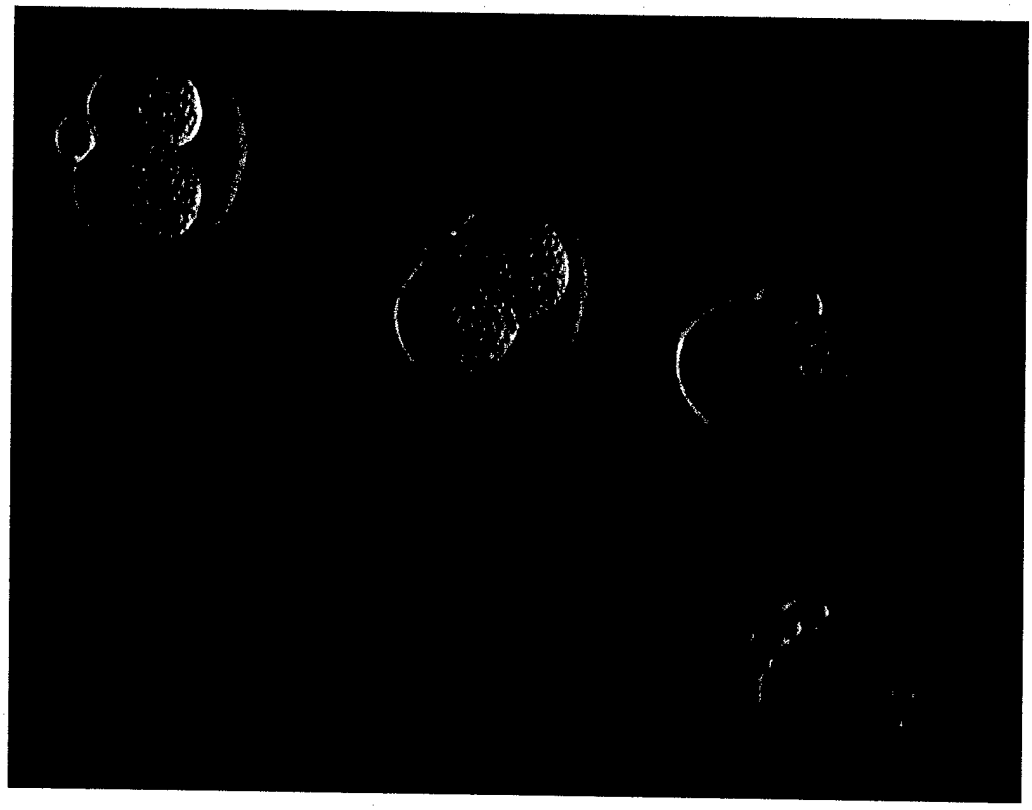

Fig. 2. Two-cell mouse embryos undergoing fusion (left to right) to produce tetraploid embryos.

sister blastomere escaped inhibition and divided into two "normal" diploid blastomeres, thus creating a three-cell mosaic. Because 4n:2n mosaics are, in some instances, viable at term, there is a possibility that reports of tetraploid embryos that survived to term (Snow, 1973) were actually 4n:2n mosaics. CB treatment also led to a low percentage of "reversions," in which cells continued to develop as diploids even though cell division appeared successfully inhibited. Although presumptive tetraploid embryos were karyotyped and shown to undergo reversion in 0.1–4% of embryos (Snow, 1975; Tarkowski et al., 1977), the numbers of 4n:2n mosaics reported are likely to underestimate the true incidence of mosaicism because the karyotyped blastocysts often had too few cells (20–30) to contain both a 4n and 2n mitotic plate (Tarkowski et al., 1977). For a description of events immediately after cytochalasin treatments, see Perry and Snow (1975).

Colchicine, a mitosis inhibitor, was one of the first methods used to induce tetraploidy in mammals, along with ethanol and heat shock applied across the oviduct (Pincus and Waddington, 1939; Beatty and Fischberg, 1952). Although initially demonstrated in the rabbit, this technology was later adopted in the mouse, either by using colchicine-treated sperm to fertilize ova or by simply injecting the uterus with colchicine during the presumptive first cleavage (Edwards, 1954). Although many polyploid animals were produced, putative polyploid zygotes occasionally extruded chromatin to the perivitelline space (McGaughey and Chang, 1969). Because this extruded material, once stained, did not resemble polar body chromatin, it was consequently postulated that zygotes of fractional ploidies had been created in addition to the more rare tri- and tetraploid ones.

Successful production of tetraploid mouse embryos has also been achieved by incubation of morulae and blastocysts in suboptimal media with transient exposure to cyclophosphamide, promoting endoduplication of the genome (Vogel and Spielmann, 1987). This procedure mainly produces triploid conceptuses, although higher multiples of the haploid chromosome number (n) can occur. Induction of specifically tetraploid embryos by cyclophosphamide is highly inefficient.

Blastomere Fusion

As it is possible to inhibit cell division to produce tetraploidy, it is also possible to fuse two diploid cells to the same effect (Fig. 2). This fusion was first demonstrated by use of inactivated Sendai virus as the agent of

fusion (Graham, 1971). The limitations of this technique are, however, that two-cell embryos must be treated individually, slowing the rate of tetraploid embryo production. Additionally, the zona pellucida must be removed before fusion, after which the manipulated embryos must be cultured two days in vitro, both of which result in lower viabilities. These, in combination with inherent toxicity of the treatment, lead to poor overall efficiency of tetraploid production (Niemierko and Opas, 1978). Not withstanding, Sendai virus-mediated tetraploidy was successfully used to describe the sex chromosome complement midgestation tetraploid embryos (O'Neill et al., 1990).

Induction of tetraploidy by polyethylene glycol (PEG) -mediated fusion was developed to create a faster and more efficient technique. Short exposures were considered critical to minimize concerns that long exposures to cytochalasin or fusogenic viruses, and not tetraploidy itself, were to blame for the aberrant development observed in treated embryos (Eglitis, 1980; Eglitis and Wiley, 1981; Spindle, 1981). An interesting refinement made by these protocols is that the technique generally involved later aggregation of two two-cell tetraploid embryos to create a 4n embryo of equal cell number to developing diploid controls. Despite this modification, it was discovered, in support of an earlier hypothesis (Smith and McLaren, 1977), that differences in cell numbers between tetra- and diploid embryos did not significantly effect the timing of developmental events (Eglitis and Wiley, 1981).

Currently, the most commonly used method of producing tetraploid embryos is electrofusion by electrical stimulation, which was first developed for fusion of plant protoplasts (Senda et al., 1979). Later, electrofusion was developed for mammalian two-cell embryos as a method for limiting exposure to toxins and controlling variability between batches of reagents used for PEG or virus-induced tetraploidy by using measurable and repeatable electrical parameters. Use of this technique was first successfully demonstrated in the mouse and rat (Berg, 1982; Kurischko and Berg, 1986). While adequately demonstrating blastomere fusion, tetraploid mammalian embryos produced by electrofusion were not proven to be capable of subsequent in vitro development until later work performed on rabbit embryos (Kubiak and Tarkowski, 1985; Ozil and Modlinski, 1986).

It is thought that during electrofusion, the electrical pulse creates a voltage difference across the plasma membranes, disrupting the closely apposed lipid membranes of the two cells. This disruption creates transient cytoplasmic bridges between the two blastomeres, which gradually expand until both cells are surrounded by one continuous plasma membrane (McLaughlin, 1993; Fig. 2). Several factors influence the efficiency of the technique. In addition to maintenance of sterile and dust-free environments, the most important factor is the alignment of the cells with respect to the electric field. If the blastomeres are oriented such that the plane of cleavage lies perpendicular to the direction of the electric field, the strongest transmembrane potential, and therefore maximal membrane disruption, develops at the point where greatest contact between the two membranes occurs (McLaughlin, 1993). Orientation of the two-cell embryos can be accomplished manually or by use of a weak alternating current field. Embryos manipulated manually may experience slightly higher fusion efficiencies (Tan et al., 1994).

Although, in principle, a single square DC pulse of 0.3–1.5 kV/cm is all that is necessary for fusion, in practice, most protocols use two to three repetitions of a short (10^{-2} sec) pulse. Fusion efficiencies of 95–100% are routinely obtainable by using commonly cited protocols (Nagy and Rossant, 1993). After blastomere fusion, embryos can be cultured to blastocyst stages with relative ease by using standard preimplantation embryo culture medium such as KSOM or M16.

To investigate whether electrofusion might produce 4n:2n mosaics, blastomeres homozygous for a ~1,000 copy mouse β-globin transgene integration (Lo, 1986) were fused (James et al., 1992). The transgenic loci were visualized by in situ hybridizations of sectioned 7.5–10.5 days postcoitum (dpc) mouse embryos. The presence of four hybridization signals per nucleus verified that conceptuses derived from electrofused blastomeres were uniformly tetraploid throughout both the embryonic and extraembryonic tissues. However, these conclusions were based on statistical analyses requiring that any diploid cells be present in at least 15% of the total number of counted nuclei. Based on comparisons with controls, and the observation that diploid cells tend to display a strong competitive advantage in 4n:2n chimeras (see below), the conclusions are likely to be valid. While electrofused blastomeres of the rat and pig are were reported to develop as homogeneously tetraploid embryos (Prather et al., 1996; Krivokharchenko et al., 2002), rabbit and bovine embryo electrofused at the two-cell stage displayed occasional (rabbit) and frequent (cow) mosaic (4n:2n) preimplantation development for unknown reasons (Ozil and Modlinski, 1986; Iwasaki et al., 1989; Curnow et al., 2000). In light of this, it may be prudent to consider the possibility that other strains of mice may not behave as those detailed above (Table 1). The electrofusion procedure, however, is considered to be robust and has since been adapted to other mammalian species as described above.

DEVELOPMENT OF THE TETRAPLOID MOUSE EMBRYO

Gene Expression in Tetraploid Embryos

A limited amount of evidence exists concerning measurements of gene products in tetraploid mammalian cells. A few observations point to the finding that tetraploidy does not result in a simple doubling of mammalian protein and RNA expression levels, a phenomenon consistent with research in plants and other species (Epstein, 1986; Osborn et al., 2003). Total RNA and malate dehydrogenase levels of tetraploid morulae

TABLE 1. Summary of Tetraploid Mouse Embryo and Chimera Studies[a]

Strain of tetraploid embryo	Defect rescued (where applicable)	Chimera production method (where applicable)	Reference
(CBA-T6T6) nuclei → (129/ter Sv × A) recipient			(Modlinski, 1978)
(CD1 × FVB/N)F1	*Ets2*	□	(Yamamoto et al., 1998)
129/Sv(GPI-1Sa/b) × C57Bl/6-JHAN(GPI1sb/b)			(Petzoldt, 1991)
A × (CBCF1)			(Tarkowski et al., 1977)
ABD2F1			(Kurischko and Berg, 1986)
B			(Clement-Sengewald and Brem, 1989)
B10CBF2	Chimerism of embryo	○	(Tarkowski et al., 2001)
B6C3F1	Comparison of aggregation and blast injection	■▲	(Peli et al., 1996)
B6CBF1	*Junb*	■	(Schorpp-Kistner et al., 1999)
			(Graham, 1971)
	Parthenogenone viability	○	(Spindle et al., 1996)
B6D2F2	*Dnmt1*	■	(Biniszkiewicz et al., 2002)
	Nuclear cloning viability	■	(Eggan et al., 2001)
	Male phenotype of ES cell-derived embryos	■	(Eggan et al., 2002)
	Nuclear cloning viability	▲	(Hochedlinger and Jaenisch, 2002)
	Nuclear cloning viability	▲	(Rideout et al., 2002)
B6JF1	*Tcf2* (*Vhnf1/Hnf1b*)	■	(Coffinier et al., 1999)
C3(B6C3F1)			(Koizumi and Fukuta, 1995)
			(Koizumi and Fukuta, 1996)
C57Bl/6	*Tbp* N Terminus	□	(Hobbs et al., 2002)
	Fosl1 (*Fra1*)	■▲	(Schreiber et al., 2000)
	Chimerism of embryo	■	(Wang et al., 1997)
CB6F1			(Sekirina et al., 1997)
CB6F2	*Pparg*	□	(Barak et al., 1999)
CBA			(Baranov, 1976)
CBB6F2	Chimerism of fetal liver	▲	(Forrester et al., 1991)
	Egfr	□	(Sibilia et al., 1998)
CCBF2			(Everett and West, 1996)
			(Everett and West, 1998)
			(Everett et al., 2000)
	Chimerism of embryo	●▲	(Nagy et al., 1990)
CCBF2 × TgMβG-1	Chimerism of embryo	●▲	(Nagy et al., 1990)
CD1	Comparison of injecting ES cells into heat-treated or 4N blasts	■	(Amano et al., 2001)
	Chimerism of fetal liver	▲	(Chambers et al., 1994)
	Hnf4	▲	(Duncan et al., 1997)
	Dsp	□	(Gallicano et al., 2001)
		○	(Goto et al., 2002)
	Extra × Chromosome	□	(Goto and Takagi, 1998)
	Ascl2 (*Mash2*)	□	(Guillemot et al., 1994)
	Brca1	□	(Hakem et al., 1996)
	Esrrb (*ErrB*)	□	(Luo et al., 1997)
	Chimerism of embryo	▲	(Misra et al., 2001)
	Chimerism of embryo	▲	(Nagy et al., 1993)
	Ascl2 (*Mash2*)	□	(Rossant et al., 1998)
	Madh4 (*Smad4/Dpc4*)	▲	(Sirard et al., 1998)
	Parthenogenone viability	○	(Spindle et al., 1996)
	Comparison of aggregation and blast injection	▲★	(Wood et al., 1993)
	Thbd (Thrombomodulin)	▲	(Isermann et al., 2001)
	Hnf4a	▲	(Li et al., 2000)

TABLE 1. (Continued)

Strain of tetraploid embryo	Defect rescued (where applicable)	Chimera production method (where applicable)	Reference
CD1 × TgMβG-1	Chimerism of embryo	●▲	(Nagy et al., 1990)
D			(Berg, 1982)
DUB:(ICR)			(Eglitis, 1980)
			(Eglitis and Wiley, 1981)
ICR	Nuclear cloning viability	■	(Amano et al., 2002)
ICR	*Bmp4*	▲	(Fujiwara et al., 2001)
			(Lu and Markert, 1980)
			(Spindle, 1981)
ICR × (B6D2F1)			(Lu and Markert, 1980)
ICR × SM/J			(Lu and Markert, 1980)
Kun-ming			(Jing-he et al., 1994)
NMRI			(Vogel and Spielmann, 1987)
			(Clement-Sengewald and Brem, 1989)
Q			(Perry and Snow, 1975)
			(Snow, 1973)
			(Snow, 1975)
			(Snow, 1976)
Q nuclei → CR or Q recipient			(Modlinski, 1981)
Rb(1.3)1Bnr × (CBBF1)			(Henery and Kaufman, 1991)
			(Henery and Kaufman, 1992)
			(Kaufman and Webb, 1990)
			(Kaufman, 1991a)
			(Kaufman, 1992)
Rb(1.3)1Bnr × (CBB6F1)			(O'Neill et al., 1990)
Rb(X,2)2Ad × (CCBF1)			(Webb et al., 1992)
Rosa26	*Vegfa*	▲	(Carmeliet et al., 1996)
	Vegfa	▲	(Carmeliet et al., 1997)
	Foxa2 (*Hnf3b*)	▲	(Dufort et al., 1998)
	Ascl2 (*Mash2*)	▲	(Tanaka et al., 1997)
Rosa26 × (BCBF1)		○	(Goto et al., 2002)
Swiss			(Graham, 1971)
			(McGaughey and Chang, 1969)
	Acvr1 (*Alk2*)	■	(Mishina et al., 1999)
	Lhx1 (*Lim1*)	■	(Shawlot et al., 1999)
Swiss × (CBB10F1)			(Kubiak and Tarkowski, 1985)
TgMβG-1 × (BCF1)		○	(James et al., 1995)
TgMβG-1 × (CCBF1)		○	(Tang and West, 2000)
TgMβG-1 × (CBBF1)			(James et al., 1992)
			(Everett and West, 1996)
			(Everett and West, 1998)
			(Everett et al., 2000)
Unspecified		○	(James et al., 1995)
			(Arman et al., 1999) Fgfr2
			(Beatty and Fischberg, 1952)
			(Berg, 1987)
			(Edwards, 1954)
			(Edwards, 1958)
	Hsp90-β	▲□	(Voss et al., 2000)

[a]Mouse strain abbreviations (International Committee on Standardized Genetic Nomenclature for Mice, 2001): A, A strains; B, C57BL; B6, C57BL/6; B10, C57B1/10; C, BALB/c; C3, C3H; CB, CBA; D1, DBA/1; D2, DBA/2; J, SJL. References for transgenic strains: TgMβG-1 (Lo, 1986), Rosa26 (Friedrich and Soriano, 1991). ■, ES cells injected into tetraploid blastocysts; ES (▲) or ICM (●) cells aggregated with tetraploid embryos; □, mutant diploid embryos aggregated with tetraploid embryos; ○, wild type diploid blastomeres aggregated with tetraploid embryos. ES, embryonic stem; ICM, inner cell mass.

are, at highest, 1.5 times that of diploid controls (Eglitis and Wiley, 1981). Additionally, fibroblast cell lines derived from human tetraploid abortuses express reduced peptidase 5 enzymatic activity when compared with diploid control lines (Schmutz and Lin, 1983).

There is also evidence to suggest that the two genomes combined during blastomere fusion may not express their genes at the same level. When two blastomeres, each bearing a unique glucose phosphate isomerase (GPI) allele on differing genetic backgrounds (129/Sv and C57Bl/6-JHan), were electrofused the ratios of GPI expression were not equivalent, suggesting that the two fused genomes exhibited different responses to tetraploidy (Petzoldt, 1991). At the morula stage, the difference in levels of the two GPI isozymes was 45:5. By blastocyst expansion, a ratio of 41:58 was observed. Although the sample sizes for a given day were small, the pooled day to day data suggested a trend toward inequality.

While studies on gene dosage in mammalian tetraploid embryos are sparse, the combined data may be taken to suggest that tetraploidy does not cause a simple doubling of the cell and all its constituents. As noted in the introduction, polyploidy is essential to the normal function of many cells in the body but is detrimental to the function of others. Although there is little data to cite regarding the mechanisms by which gene dosage is regulated in polyploid mammalian cells, clearly, these cells must possess a genetic mechanism for alleviating the negative consequences of polyploidy. The most parsimonious explanation for the appearance of polyploid species (e.g., *T. barrerae*) and the rare observation of late gestation polyploid mammals is that within the gene pool from which each of these organisms were derived there exists a combination of alleles which favor resistance to polyploidy.

Cellular Effects of Tetraploidy

Tetraploidy has several documented effects on individual cells in the conceptus, including cell size, the length of the cell cycle, and consequently, the total cell number in the tetraploid embryo. Tetraploid embryos likely have a slower cell cycle and consequently fewer cells than their age-matched diploid counterparts. However, the size of an entire tetraploid embryo remains roughly equivalent to stage-matched diploid controls. Indeed, the earliest reports of tetraploidy in mice described an essentially inversely proportional relationship between ploidy and cell count (Beatty and Fischberg, 1951; Edwards, 1958). This relationship appears true from cleavage stages through organogenesis (Henery et al., 1992).

Although it was reported previously that cell cycles were unaffected in preimplantation tetraploid embryos (Henery and Kaufman, 1991), a more recent report calculated the cell cycles of preimplantation tetraploids to be approximately 2 hr slower than controls (Koizumi and Fukuta, 1995). Although it is uncertain what led to the discrepancy between these findings, one caveat to these studies is a lack of a reliable method for stage matching 4n and 2n embryos, as these studies relied primarily on measurements that were taken at specified times after ovulation. In this case, it cannot be ensured that the 4n and 2n embryos were truly stage-matched.

The total number of cells in tetraploid blastocysts was reported at 22, whereas diploid blastocysts averaged 69 cells in the same study (Koizumi and Fukata, 1995). These numbers concur with other observations in several strains of mice (Snow, 1976; Tarkowski et al., 1977; Baranov, 1983; referenced in Dyban and Baranov, 1987). Although cell numbers were different in similarly aged tetraploid and diploid preimplantation embryos, they underwent compaction and blastulation at equivalent times (Koizumi and Fukuta, 1996). Differences in cell number but not cell cycle times were observed between tetraploid and diploid rat embryos (Krivokharchenko et al., 2002).

Whereas cell counts are reported as depressed, there is no observation that overall preimplantation size is decreased. This finding may be because tetraploid cells generally have a greater volume than diploid cells (Snow, 1975, 1976; Tarkowski et al., 1977; Niemierko and Opas, 1978). The nucleated blood cells of the visceral yolk sac were estimated to be four times the volume of their diploid counterparts and may be the cause of the hemorrhages observed in tetraploid yolk sacs perhaps because the larger cells must be pushed through vasculature of diploid diameters (Snow, 1975). This expansion of cell volume is also observed in tetraploid human fibroblasts (Chang et al., 1983; Schmutz and Lin, 1983). This finding is reasonable, because naturally polytene and polyploid cells are often dramatically increased in cell volume (e.g., trophoblast giant cells, or liver parenchymal cell). Fetal nucleated blood cells have been examined for the effects of tetraploidy (Henery and Kaufman, 1992). This cell was chosen largely due to the spheroidal morphology of nucleated blood cells, which facilitated morphometrics. Nuclear and cellular volumes were calculated under the assumption that an average cell diameter would be less than the section thicknesses. The findings were that nucleic and cellular volumes of tetraploid cells between 8.25 and 14.5 dpc were on average 1.7–2.3, nearly the theoretical 2, times that of diploid controls. Of interest, histologic sections of tetraploid embryos revealed no significant effects of cell size on histology in regions other than the forebrain and branchial arch with the exception of larger cell size (Kaufman, 1992). Normal histology was documented in tetraploid germ cells, gonads (Kaufman, 1991a), and kidneys (Kaufman, 1992).

Developmental Potential of Tetraploid Mouse Embryos

The longest reported survival of tetraploid mouse embryos were documented by Snow (1973, 1975). By using CB-treated outbred Q strain mouse embryos, four of 78 (5.1%) implantations survived to late term development, three of which were born. The fourth mouse was dissected at 17.5 dpc and had externalized viscera. By using similar techniques,

Tarkowski et al. (1977) were unable to produce embryos beyond the tenth day of development. Most embryos began manifesting defects on the eighth day, including limited neural plates and "scanty" mesoderm. Similar defects were also detected in the rabbit (Ozil and Modlinski, 1986). By using ((CBA × C57Bl/6)F1 × A) hybrids, approximately 4% of CB-treated zygotes "reverted" to diploid chromosome counts (Tarkowski et al., 1977). This finding is proportionately similar to Snow's 3 term fetuses of 112 transferred blastocysts. Tarkowski, in fact, noted that as many as 20% of his embryos developed as mosaics, although by the tenth day, the tetraploid cells were virtually eliminated from the embryos, presumably due to a competitive disadvantage between the tetraploid and diploid cells.

Tetraploid embryo survival, although at low frequency (1.7–4.2%), of up to 14 and 15 dpc was described in backgrounds that include at least one (C57Bl × CBA)F1 parent (Kaufman and Webb, 1990; Kaufman, 1991a, b, 1992; Henery and Kaufman, 1992). These later stages were demonstrated to be homogenously tetraploid (Kaufman and Webb, 1990; James et al., 1992). Although during preimplantation development there was no marked difference in embryonic size, midgestation tetraploid embryos were generally 85% of the size of diploid controls (Henery et al., 1992). The defects in these embryos were most notable in the characteristic morphologies of the forebrain and aberrant or absent eyes, resembling holoprosencephaly. These defects could be results of abnormal migrations of prechordal mesoderm or neural crest (Kaufman, 1991b). This hypothesis was supported by the occasional appearance of forked or deviated neural tubes (Kaufman and McLaren, 1992). Malformations of the vertebral axis and heart were also evident as well as occasional *situs inversus* and absence of the pituitary gland (Kaufman and Webb, 1990; Kaufman, 1991b, 1992). Additionally, the combination of the use of CB and that the previously described full-term tetraploid embryos (Snow, 1973, 1975) did not display these characteristic features in toto led to the suggestion, once again, that viable "tetraploids" may have been 4n:2n mosaics (Henery et al., 1992). Snow (1975) reported reduced brain weight and deformities of the eye, reminiscent of those observed in Kaufman's homogenous tetraploids. However, the associated defects did not always display the bilateral symmetry that were generally evident in the later tetraploid embryos. Although the true status of the late gestation embryos reported by Snow remains the subject of controversy, it must be stated that karyotypes of the embryonic and extraembryonic tissue appeared to rule out 4n:2n chimerism (Snow, 1975). To date, the major objection to Snow's claim is that it has been so far unrepeatable. It is perhaps interesting to note that not all of the Snow embryos could be sexed chromosomally (Snow, 1975). One speculation might be that, if conclusions were being drawn from chromosomal spreads of limited quality, it might have been possible to score embryos as tetraploid when, in fact, the true chromosomal complement may have been better described as "near-tetraploid." The controversy surrounding these embryos is not likely to be resolved until a new report of term tetraploid mice emerges.

No significant defects of the gonads have been detected in tetraploid embryos of either sex (Kaufman, 1991a). Primordial germ cells in both XXYY and XXXX animals were observed at day 11, although some delay in the migration of these cells from the allantois to the hindgut was evident. Additionally, tetraploid embryos produced by either electrofusion (Kaufman, 1991a) or viral fusion (O'Neill et al., 1990) showed observable bias in the incidence of XXYY and XXXX embryos at somite stages. Later data from 13–14.5 dpc embryos allowed the observation of both sexes morphologically, albeit in a small sample (n = 6 females, 8 males; Kaufman, 1991a, b, 1992). With respect to sex chromosome distributions in human tetraploid embryos, there were roughly 50% more XXXX tetraploid embryos than XXYY in human spontaneous abortions (Sheppard et al., 1982; Surti et al., 1986). It is hypothesized that, although the differences between human and mouse may be true species differences, they may also be due to inclusion of 4n:2n mosaic numbers within the human spontaneous abortion studies (Kaufman, 1991a).

Surprisingly, a rigorous description of extraembryonic phenotypes associated with tetraploidy is lacking in the literature. It is known that placental weights of 4n:2n chimeric animals are significantly greater than in diploid controls (James et al., 1995), but the reasons for this difference are unknown. Given the frequent use of tetraploid embryo complementation (described later), this would seem a significant gap in knowledge in the field.

Although mouse sexes appear in equivalent numbers, the incidence of vascular abnormalities may be biased. While four XXYY embryos examined displayed vascular abnormalities, the three XXXX embryos in the same study displayed no such defects (Kaufman, 1992). In other studies, X inactivation appeared to occur proportionately in embryonic and mesodermal tissues with two X chromosomes of XXXX mice inactivating without bias toward parent of origin at 10.5 dpc. However, in the endodermal tissues preferential inactivation of paternal X chromosomes was observed. In XXYY embryos, only infrequent inactivation was observed (Webb et al., 1992). It is possible, however, that some classes of inactivation may not have been observed if they were selected against during the 10 days between tetraploid induction and analysis.

Maximal development of tetraploid embryos is likely to be strain dependent, although this has yet to be rigorously documented. A summary of the strains that have been used in the production of tetraploid embryos and their chimeras is provided in Table 1. The most advanced development of tetraploid embryos was reported in (C57Bl x CBA)F1 hybrid females mated to Rb(1.3)Bnr males (unpublished evidence in Kaufman, 1991a), or outbred Q strain (Snow, 1973, 1975, 1976). In each of these cases, hybrid strains were used and advanced development was observed only rarely. Together, these facts suggest a polygenic mechanism governing fetal tolerance to tetraploidy. In this model, an occasional

embryo is produced that possesses a set of alleles from the gene pool, which confers a degree of fetal tolerance to tetraploidy.

Tissue-Specific Effects of Tetraploidy: 4n:2n Mouse Chimeras

Although 4n:2n mosaicism has hampered the ability to produce pure tetraploid embryos, the intentional production of 4n:2n mouse chimeras has led to a powerful modern developmental biology technique. Tetraploid embryo complementation allows the rescue of extraembryonic phenotypes. This process occurs by taking advantage of the tendency of diploid, particularly ES, cells to preferentially colonize embryonic tissues when associated with tetraploid embryos (Beddington and Robertson, 1989). Although conditional knock-outs could be used to the same end, only mice expressing Cre recombinase in an epiblast-specific manner have been reported so far (Tallquist and Soriano, 2000). To date, there are no reports of trophoblast or visceral endoderm-specific Cre-expressing mice. The tendency of ES cells to contribute to the embryonic lineages combined with tetraploid complementation of extraembryonic phenotypes may also be exploited to allow the production of embryos nearly 100% derived from ES cells (Nagy et al., 1993). Although developed initially in the mouse, this technique has now been adopted in cattle (Iwasaki et al., 1999, 2000) and pigs (Prather et al., 1996).

Blastocyst Injection vs. Aggregation for Making 4n:2n Chimeras

Chimeras can be produced by aggregation of two separate embryos or injection of foreign cells directly into early embryos. The blastocyst injection method is perhaps the most direct method of producing a chimera. The reasons for using one technique over the other are arguable, but may end up being simply a matter of preference between laboratories. Indeed, production of chimeric mice from low-passage ES cells show little measurable difference between the two techniques when chimeras are analyzed at midgestation (Peli et al., 1996) or by their ability to "go germline" (Wood et al., 1993). Blastocyst injection, however, may be better suited for producing chimeras from ES cells of high passage numbers (Wang et al., 1997).

Blastocyst injection does require the (sometimes difficult) manipulation of "sticky" and more flexible tetraploid blastocysts. The reasons behind the differences in texture are probably several fold. First, tetraploid blastocysts have fewer cells with which to establish cell–cell adhesions. Second, a doubling of the volume of a sphere does not cause a doubling of the surface area. Volume and area are related by a factor of r/3 (where r is the radius of the sphere). Given average cell size data (Henery and Kaufman, 1992), one can estimate the increase in surface area of a (red blood) cell due to a 100% increase in volume as an increase of merely 51%. This finding is similar to an unreferenced claim that a doubling in volume results in a 59% increase in surface area (Snow, 1975). As such, the fewer cells in the tetraploid blastocyst may have a reduced ability to adhere to one another than their smaller, more numerous diploid counterparts. Third, tetraploid embryos are generally cultured to blastocyst stage in vitro for 2–3 days before injection. This strategy may also cause fundamental changes in the texture of the embryo relative to the freshly harvested diploid blastocysts commonly used for blastocyst injections. There is evidence, however, that 4n:2n chimeras produced by blastocyst injection may be more viable than those produced by aggregation (Wang et al., 1997). Additionally, blastocyst injection allows one to grade and individually select ES cells based on their morphology. Because aggregations are performed with clumps of ES cells, this selection is not possible.

The use of aggregation chimeras appears to be slightly more popular than injection chimeras for the production of 4n:2n chimeras. The reasons argued for this choice are that aggregation chimeras can be produced at 100–150 chimeras per hour vs. 20–30 by means of blastocyst injection (Wood et al., 1993), aggregation chimeras do not require the elaborate microinjection apparatus and require far less practice before proficiency of the technique is achieved. The argument is then extended that the greater number of chimeras produced by aggregation compensates for any reduced viabilities relative to blastocyst injection. As described earlier, blastocyst cavity formation of tetraploid embryos is slightly delayed with respect to those of diploid mice. Depending on the light cycle of the animal facility and consequently the time of fertilization, blastocyst injections of tetraploid embryos may require injections to be performed at inconvenient times.

Development of 4n:2n Chimeras

A homogenously tetraploid mouse embryo is generally capable of development to the pregastrula and sometimes later stages. Chimeras of 4n and 2n embryos often survive to term. In these chimeras, the tetraploid cells are underrepresented in the embryo proper due to a selective disadvantage with the 2n cells. The first 4n:2n mosaic mouse was produced by exposure of two- to four-cell embryos to CB (Tarkowski et al., 1977). In these embryos, it was noted that a very low proportion of tetraploid cells were present in the embryo proper (<4%), but up to a 50% contribution was evident in the extraembryonic tissues. To better control the production of the chimera, 4n:2n chimeras were later created by using two wild-type two-cell embryos aggregated with a CB-induced two-cell tetraploid embryo. This strategy resulted in two live-born 4n:2n chimeras out of 59 transferred embryos (Lu and Markert, 1980). Although the extraembryonic tissues could not be studied, an approximately 3% contribution of the tetraploid cells to the bone marrow of one chimera was reported. This finding suggested that, under some conditions, tetraploid cells may be retained postnatally.

The mechanism by which tetraploid cells are primarily restricted to the extraembryonic tissues is largely unknown. There are probably many factors causing low contribution of tetraploid cells to the embryo, including issues of fitness, compromised developmental potential, or possibly differential rates of cell division. The extraembryonic tissues possess a large number of naturally polyploid cells, including trophoblast giant and syncytiotrophoblast cells. As the function of these cells includes resistance to detrimental effects of polyploidy, experimentally induced tetraploid cells are likely to be healthiest in those tissues that have evolved to tolerate polyploidy. There is circumstantial data to support this idea in other areas of the body, including those derived from the embryo-proper. For instance, the same theory may explain why experimentally produced tetraploid cells persist in the developing liver of midgestation 4n:2n chimeric mice (Goto et al., 2002). Alternatively, there is also evidence to suggest that large cells may show a tendency to colonize trophectodermal lineages regardless of ploidy (Tang and West, 2000). In this case, it is hypothesized that large cells are mechanically forced to the outer edges of compacting morula and consequently adopt extraembryonic fates (Everett and West, 1996; Tang and West, 2000). Whatever the cause, these factors probably begin acting quite soon after chimera formation to bias tissue types toward one ploidy or the other. The tendency of tetraploid cells to colonize the trophectoderm occurs at the blastocyst stage. This colonization may be accompanied by selection against tetraploid cells in the ICM, although at 4.5 days of development, the tetraploid cells may still be present at levels of 13% (Everett and West, 1996, 1998; Everett et al., 2000). At days 7.5, 10.5, 12.5, 13.5, 16.5, and 17.5 of development, the tetraploid cells are observed to contribute to progressively smaller amounts of the ICM-derived tissues (Tarkowski et al., 1977; Nagy et al., 1990; James et al., 1995; Wang et al., 1997; Tang and West, 2000; Goto et al., 2002). At 12.5 days of gestation, a minor contribution of tetraploid cells to the embryo proper was observed in small pockets of the heart, liver, and skin (Goto et al., 2002). In five of 60 documented newborn or older mice, tetraploid contribution was determined by karyotype or GPI assays at levels of 3–50% in the heart, blood, lung, and liver; tetraploidy could not be detected in any of the remaining 55 mice (Lu and Markert, 1980; Nagy et al., 1993; Wang et al., 1997). Again, the developmental potential of the tetraploid cells is thought to be strain dependent and may exhibit different degrees of contribution in different genetic backgrounds.

A potential caveat to the pattern of development of tetraploid cells in 4n:2n chimeras is that adult cells, when allowed to spontaneously fuse with ES cells, produced cell lines with tetraploid or near-tetraploid karyotypes (Matveeva et al., 1998; Terada et al., 2002; Ying et al., 2002). Two studies of fusion adult cells found high incidence of loss of chromosomes after the process of fusion (Matveeva et al., 1998; Terada et al., 2002). One set of ES cell fusion products was not able to produce chimeras when injected into diploid blastocysts (Terada et al., 2002). Another near tetraploid cell line exhibited instability, losing nearly half of its chromosomes over five to seven passages (Matveeva et al., 1998). In one case, however, ES cell fusion products were capable of forming intestinal, renal, cardiac, and hepatic cells after injection of the cells into diploid blastocysts (Ying et al., 2002). Karyotypes of the cell lines generated in the study revealed a "tetraploid or near-tetraploid" (Ying et al., 2002) complement of chromosomes, although it is unclear which cell line produced the chimeras, as well as how many passages elapsed between the karyotyping and blastocyst injection (Ying et al., 2002). Because the developmental potential of the ES cell fusion products was presented without reference to the particular karyotype of the cell line used, and without demonstration of maintenance of ploidy in the resulting cell lines or chimeras it is not possible to compare the results with other results cited in this review.

Types of 4n:2n Chimeras

Primarily, three types of chimeras are made using tetraploid embryos. 4n:2n chimeras may be produced by combining 4n embryos with (1) 2n embryos, (2) ES cells, or (3) inner cell mass cells (Fig. 3; page 763). In each case, the tetraploid cells contribute primarily in the extraembryonic tissues. However, the cell distributions are somewhat modified by the type of chimera generated.

Aggregation of tetraploid morulae with diploid morulae has often been used in tetraploid embryo complementation experiments to segregate the phenotypic effects of a given genetic background to embryonic rather than extraembryonic tissues by aggregation of a mutant embryo with a tetraploid wild-type embryo. This technique was used successfully to circumvent extraembryonic lethality to produce adult homozygous mutant mice as well as to study the extraembryonic defects themselves (Guillemot et al., 1994). In other studies, this technique was used to bypass extraembryonic defects to study additional embryonic defects (Luo et al., 1997; Rossant et al., 1998; Sibilia et al., 1998; Yamamoto et al., 1998; Li et al., 2000; Gallicano et al., 2001; Hobbs et al., 2002). Diploid embryos aggregated with tetraploid embryos will display the greatest degree of developmental potential, in this case, the diploid cells will be expected to contribute to both embryonic and extraembryonic lineages.

Rather than aggregations of tetraploid and diploid morulae, chimeric embryos of two-cell tetraploid embryos and diploid ES cells have also been produced (Fig. 3; Nagy et al., 1990, 1993). Due to the limited ability of ES cells to colonize trophoblast (Beddington and Robertson, 1989), the segregation of diploid and tetraploid cells in these chimeras is more evident. In saying this, the reported studies of cell distributions have been exclusively postgastrulation and have been derived from a variety of mouse strains. Thus, a major unresolved question is how cells are distributed between implantation and 7.5 days of development. Although a recent study reported nearly equivalent numbers and dis-

tribution of tetraploid and diploid cells within gastrulation-staged chimeras produced by aggregation of embryos (Goto et al., 2002), this assessment has not yet been extended to chimeras produced from ES cells. Having said this, aggregation or injection of ES cells into otherwise wild-type tetraploid embryos has been used for many of the same types of experiments as 4n:2n embryo aggregation chimeras (Fig. 3). It has also been used to produce mutant embryos directly from ES cells for analysis of their mutant phenotype without generating animals through matings of germline chimeras. This strategy proved particularly useful in the case of the heterozygous embryonic lethal *Vegf* allele. Production of 4n:ES cell chimeras allowed the analysis of embryonic defects in both heterozygous and homozygous mutant embryos without the need for targeted conditional alleles (Carmeliet et al., 1996, 1997). Although most mutations are not complicated by heterozygous lethality, other benefits of this approach include the rapid access to the embryonic phenotype as well as the inexpense of producing mutants without breeding. It is also possible to speed up the traditional breeding process with tetraploid embryos by creating chimeras from targeted ES lines that have spontaneously lost the Y chromosome. Because XO females are viable and fertile, chimeras produced by this technique can be mated to normal male chimeras from the same targeted (XY) ES line to more quickly produce homozygous mutant offspring (Eggan et al., 2002).

ICM aggregation is similar to aggregation with ES cells. In this case, ICMs are grown as in the first steps of isolating ES cells. Rather than passaging these cells, however, they are disaggregated with protease and combined directly with the tetraploid embryos. This produces a population of cells of heterogeneous embryonic developmental potentials, including a subset of pluripotent "ES-like" cells. This was initially used as a control for the original study describing 4n:ES aggregation chimeras. Notably, 4n:ICM cell aggregations produced a greater percentage of healthy neonates than 4n:ES cell chimeras (Nagy et al., 1990). This difference in fitness may be an indication that the health of chimeras produced by aggregation is likely due to differences in donor cells rather than problems inherent to the aggregation technique (Nagy and Rossant, 2001). Recently, this type of chimera was produced to show that the low viability of embryos cloned by nuclear transplantation is not solely because of failures of cloned trophectoderm but also due to suboptimal developmental potential of the ICM (Amano et al., 2002).

PERSPECTIVES

The production of tetraploid embryos has become a useful tool for mouse developmental biologists within the past decade and is increasingly being used in other mammalian systems. The phenomenon by which tetraploid and diploid cells are segregated in chimeras has been exploited in numerous examples to rescue extraembryonic defects, compartmentalize genetically dissimilar tissues, and increase the rapidity by which transgenic mice are created and their phenotypes analyzed. Questions remain, however, concerning the effects of genetic background on the developmental potential of tetraploids and their chimeras as well as questions concerning the distribution of tetraploid and diploid cells in chimeras, especially in peri-implantation and streak stage embryos. Particularly for tetraploid embryo:ES cell chimeras, the strains used are often not the strains in which the limits of the tetraploid embryo complementation technique itself was originally defined. This finding leads to the concern that the discrete compartmentalization of diploid and tetraploid cells, as originally described may not hold true in other mouse strains. The level of concern is, of course, dependent on a couple of factors. The first is that later staged embryos are less likely to be affected by these strain concerns due to the competitive disadvantage shown by tetraploid cells. The idea is that "contaminating" tetraploid lineages within the embryo proper are likely to constitute progressively less of the embryo as age progresses. The second consideration concerns the nature of the experiment itself. For example, consider an experiment in which a non-cell autonomous mutation causing extraembryonic lethality is rescued by tetraploid embryo complementation to study the defect in the embryo proper. Especially at earlier stages, this experiment risks being affected by the presence of "contaminating" tetraploid cells in the embryo proper. In this case, it is plausible that the embryo may not exhibit the phenotype expected of a truly mutant embryo, due to the supply of wild-type allele in the contaminating tetraploid cells. Conversely, the same experiment involving a cell-autonomous defect may not be greatly affected by small amounts of contaminating tetraploid cells in the embryo proper. In these cases, controls for the contribution of tetraploid cells to the embryonic lineage are essential to understanding the limitations of a tetraploid complementation experiment.

While being widely blamed for variability in results, the issue of genetic variability with respect to tetraploid development has not been explored in the literature beyond a single unpublished result describing maximal tetraploid developmental potential in (C57Bl x CBA)F1 hybrid females mated to Rb(1.3)Bnr males when compared against other unspecified strains (Kaufman, 1991a). As much as it is of practical importance to evaluating results of tetraploid complementation, the issue of genetic background differences in the developmental potential of tetraploid cells is also of academic interest. Because genetics can apparently greatly influence tetraploid embryo viability, it would seem very interesting to understand which genes regulate this process. At a molecular level, it remains unclear what factors allow tissues to be resistant or sensitive to polyploidy. A conceptually simple, if logistically challenging, method of mapping the loci that promote sensitivity or resistance to tetraploidy could be developed by analyzing the progeny of interspecific or recombinant inbred strains for tetraploid developmental potential. To do this, two strains of mice would

need to be documented to display differing developmental potential. Perhaps successful tetraploidy will require a specific balance of genes governing a variety of cellular systems. Gene balance is an important consequence of genomic imprinting in mammals and, thus, may also be disturbed in tetraploid embryos.

This review has attempted to summarize the history of mammalian experimental tetraploidy and outline some of the benefits and caveats to the technologies that have arisen from it. Despite concerns, experimental tetraploidy continues to be a powerful technique and continues to pave new roads for mammalian research.

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REFERENCES

Amano T, Kato Y, Tsunoda Y. 2001. Comparison of heat-treated and tetraploid blastocysts for the production of completely ES-cell-derived mice. Zygote 9: 153–157.

Amano T, Kato Y, Tsunoda Y. 2002. The developmental potential of the inner cell mass of blastocysts that were derived from mouse ES cells using nuclear transfer technology. Cell Tissue Res 307: 367–370.

Arman E, Haffner-Krausz R, Gorivodsky M, Lonai P. 1999. Fgfr2 is required for limb outgrowth and lung-branching morphogenesis. Proc Natl Acad Sci U S A 96:11895–11899.

Barak Y, Nelson MC, Ong ES, Jones YZ, Ruiz-Lozano P, Chien KR, Koder A, Evans RM. 1999. PPAR gamma is required for placental, cardiac, and adipose tissue development. Mol Cell 4: 585–595.

Baranov VS. 1976. Spontaneous polyploidy in laboratory mice. Embryological and cytogenetic analysis. Ontogenez 7:229–238.

Baranov VS. 1983. Chromosomal control of early embryonic development in mice. I. Experiments on embryos with autosomal monosomy. Genetica 61: 165–177.

Beatty R, Fischberg M. 1951. Cell number in haploid, diploid and polyploid mouse embryos. J Exp Biol 28:541–552.

Beatty R, Fischberg M. 1952. Heteroploidy in mammals. II. Induction of tetraploidy in pre-implantation mouse eggs. J Genet 50:471–479.

Beddington RS, Robertson EJ. 1989. An assessment of developmental potential of embryonic stem cells in the midgestation mouse embryo. Development 105:733–737.

Berg H. 1982. Biological implications of electric field effects. Part V. Fusion of blastomeres and blastocysts of mouse embryos. Bioelectrochem Bioenerg 9: 223–228.

Berg H. 1987. Electrotransfection and electrofusion of cells and electrostimulation of their metabolism. Stud Biophys 119:17–29.

Biniszkiewicz D, Gribnau J, Ramsahoye B, Gaudet F, Eggan K, Humpherys D, Mastrangelo MA, Jun Z, Walter J, Jaenisch R. 2002. Dnmt1 overexpression causes genomic hypermethylation, loss of imprinting, and embryonic lethality. Mol Cell Biol 22:2124–2135.

Brodsky V, Uryvaeva I. 1985. Genome multiplication in growth and development: biology of polyploid and polytene cells. Cambridge: Cambridge University Press.

Bromhall JD. 1975. Nuclear transplantation in the rabbit egg. Nature 258:719–722.

Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380:435–439.

Carmeliet P, Moons L, Dewerchin M, Mackman N, Luther T, Breier G, Ploplis V, Muller M, Nagy A, Plow E, Gerard R, Edgington T, Risau W, Collen D. 1997. Insights in vessel development and vascular disorders using targeted inactivation and transfer of vascular endothelial growth factor, the tissue factor receptor, and the plasminogen system. Ann N Y Acad Sci 811:191–206.

Carr D. 1972. Chromosomal anomalies in human foetuses. Res Reprod 4:3–4.

Chambers CA, Kang J, Pawling J, Huber B, Hozumi N, Nagy A. 1994. Exogenous Mtv-7 superantigen transgene expression in major histocompatibility complex class II I-E- mice reconstituted with embryonic stem cell-derived hematopoietic stem cells. Proc Natl Acad Sci U S A 91:1138–1142.

Chang PL, Georgiadis N, Joubert GI, Davidson RG. 1983. Gene dosage effects in human diploid and tetraploid fibroblasts. Exp Cell Res 145:277–284.

Clement-Sengewald A, Brem G. 1989. Electrofusion parameters for mouse two-cell embryos. Theriogenology 32: 159–169.

Coffinier C, Thepot D, Babinet C, Yaniv M, Barra J. 1999. Essential role for the homeoprotein vHNF1/HNF1 beta in visceral endoderm differentiation. Development 126:4785–4794.

Contreras LC, Torres-Mura JC, Spotorno AE. 1990. The largest known chromosome number for a mammal, in a South American desert rodent. Experientia 46:506–508.

Creasy MR, Crolla JA, Alberman ED. 1976. A cytogenetic study of human spontaneous abortions using banding techniques. Hum Genet 31:177–196.

Curnow EC, Gunn IM, Trounson AO. 2000. Electrofusion of two-cell bovine embryos for the production of tetraploid blastocysts in vitro. Mol Reprod Dev 56: 372–377.

Davies GE, Howard CM, Farrer MJ, Coleman MM, Bennett LB, Cullen LM, Wyse RK, Burn J, Williamson R, Kessling AM. 1995. Genetic variation in the COL6A1 region is associated with congenital heart defects in trisomy 21 (Down's syndrome). Ann Hum Genet 59(Pt 3):253–269.

Dufort D, Schwartz L, Harpal K, Rossant J. 1998. The transcription factor HNF3 beta is required in visceral endoderm for normal primitive streak morphogenesis. Development 125:3015–3025.

Duncan SA, Nagy A, Chan W. 1997. Murine gastrulation requires HNF-4 regulated gene expression in the visceral endoderm: tetraploid rescue of Hnf-4(-/-) embryos. Development 124:279–287.

Dyban A, Baranov V. 1987. Cytogenetics of mammalian embryonic development. New York: Oxford University Press.

Edwards RG. 1954. Colchicine-induced heteroploidy in early mouse embryos. Nature 174:276–277.

Edwards RG. 1958. Colchicine-induced heteroploidy in the mouse. II. The induction of tetraploidy and other types of heteroploidy. J Exp Zool 137:349–362.

Eggan K, Akutsu H, Loring J, Jackson-Grusby L, Klemm M, Rideout WM III, Yanagimachi R, Jaenisch R. 2001. Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. Proc Natl Acad Sci U S A 98:6209–6214.

Eggan K, Rode A, Jentsch I, Samuel C, Hennek T, Tintrup H, Zevnik B, Erwin J, Loring J, Jackson-Grusby L, Speicher MR, Kuehn R, Jaenisch R. 2002. Male and female mice derived from the same embryonic stem cell clone by tetraploid embryo complementation. Nat Biotechnol 20:455–459.

Eglitis MA. 1980. Formation of tetraploid mouse blastocysts following blastomere fusion with polyethylene glycol. J Exp Zool 213:309–313.

Eglitis MA, Wiley LM. 1981. Tetraploidy and early development: effects on developmental timing and embryonic metabolism. J Embryol Exp Morphol 66:91–108.

Epstein C. 1986. The consequence of chromosome imbalance: principles, mechanisms, and models. Cambridge: Cambridge University Press.

Everett CA, West JD. 1996. The influence of ploidy on the distribution of cells in chimaeric mouse blastocysts. Zygote 4: 59–66.

Everett CA, West JD. 1998. Evidence for selection against tetraploid cells in tetraploid←→diploid mouse chimae-

ras before the late blastocyst stage. Genet Res 72:225–228.
Everett CA, Stark MH, West JD, Davidson D, Baldock RA. 2000. Three-dimensional reconstruction of tetraploid←→diploid chimaeric mouse blastocysts. J Anat 196(Pt 3):341–346.
Forrester LM, Bernstein A, Rossant J, Nagy A. 1991. Long-term reconstitution of the mouse hematopoietic system by embryonic stem cell-derived fetal liver. Proc Natl Acad Sci U S A 88:7514–7517.
Friedrich G, Soriano P. 1991. Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. Genes Dev 5:1513–1523.
Fujiwara T, Dunn NR, Hogan BL. 2001. Bone morphogenetic protein 4 in the extraembryonic mesoderm is required for allantois development and the localization and survival of primordial germ cells in the mouse. Proc Natl Acad Sci U S A 98:13739–13744.
Gallardo MH, Bickham JW, Honeycutt RL, Ojeda RA, Kohler N. 1999. Discovery of tetraploidy in a mammal. Nature 401: 341.
Gallicano GI, Bauer C, Fuchs E. 2001. Rescuing desmoplakin function in extraembryonic ectoderm reveals the importance of this protein in embryonic heart, neuroepithelium, skin and vasculature. Development 128:929–941.
Goto Y, Takagi N. 1998. Tetraploid embryos rescue embryonic lethality caused by an additional maternally inherited X chromosome in the mouse. Development 125:3353–3363.
Goto Y, Matsui J, Takagi N. 2002. Developmental potential of mouse tetraploid cells in diploid ⟷ tetraploid chimeric embryos. Int J Dev Biol 46:741–745.
Graham C. 1971. Virus assisted fusion of embryonic cells. In: Karolinska Symposia on Research Methods in Reproductive Biology. 3rd Symposium. January 25–27, 1971. In vitro methods in reproductive cell biology. Diczfalusy E, Ed., Stockholm. p 154–167.
Guillemot F, Nagy A, Auerbach A, Rossant J, Joyner AL. 1994. Essential role of Mash-2 in extraembryonic development. Nature 371:333–336.
Hakem R, de la Pompa JL, Sirard C, Mo R, Woo M, Hakem A, Wakeham A, Potter J, Reitmair A, Billia F, Firpo E, Hui CC, Roberts J, Rossant J, Mak TW. 1996. The tumor suppressor gene Brca1 is required for embryonic cellular proliferation in the mouse. Cell 85:1009–1023.
Hassold T, Hunt P. 2001. To err (meiotically) is human: the genesis of human aneuploidy. Nat Rev Genet 2:280–291.
Hassold T, Chen N, Funkhouser J, Jooss T, Manuel B, Matsuura J, Matsuyama A, Wilson C, Yamane JA, Jacobs PA. 1980. A cytogenetic study of 1000 spontaneous abortions. Ann Hum Genet 44:151–178.
Henery C, Kaufman MH. 1991. Cleavage rates of diploid and tetraploid mouse embryos during the preimplantation period. J Exp Zool 259:371–378.
Henery CC, Kaufman MH. 1992. Relationship between cell size and nuclear volume in nucleated red blood cells of developmentally matched diploid and tetraploid mouse embryos. J Exp Zool 261:472–478.
Henery CC, Bard JB, Kaufman MH. 1992. Tetraploidy in mice, embryonic cell number, and the grain of the developmental map. Dev Biol 152:233–241.
Hobbs NK, Bondareva AA, Barnett S, Capecchi MR, Schmidt EE. 2002. Removing the vertebrate-specific TBP N terminus disrupts placental beta2m-dependent interactions with the maternal immune system. Cell 110:43–54.
Hochedlinger K, Jaenisch R. 2002. Monoclonal mice generated by nuclear transfer from mature B and T donor cells. Nature 415:1035–1038.
International Committee on Standardized Genetic Nomenclature for Mice. Chairperson: Jackson I. 2001. Rules for nomenclature of mouse and rat strains. Available at: http://www.informatics.jax.org/mgihome/nomen/strains.shtml.
Isermann B, Hendrickson SB, Hutley K, Wing M, Weiler H. 2001. Tissue-restricted expression of thrombomodulin in the placenta rescues thrombomodulin-deficient mice from early lethality and reveals a secondary developmental block. Development 128:827–838.
Iwasaki S, Kono T, Fukatsu H, Nakahara T. 1989. Production of bovine tetraploid embryos by electrofusion and their developmental capability in vitro. Gamete Res 24:261–267.
Iwasaki S, Ito Y, Iwasaki S. 1999. In vitro development of aggregates of bovine inner cell mass cells or bovine mammary cells and putative tetraploid embryos produced by electrofusion. J Reprod Dev 45:65–71.
Iwasaki S, Campbell KH, Galli C, Akiyama K. 2000. Production of live calves derived from embryonic stem cells aggregated with tetraploid embryos. Biol Reprod 62:470–475.
James RM, Kaufman MH, Webb S, West JD. 1992. Electrofusion of mouse embryos results in uniform tetraploidy and not tetraploid/diploid mosaicism. Genet Res 60: 185–194.
James RM, Klerkx AH, Keighren M, Flockhart JH, West JD. 1995. Restricted distribution of tetraploid cells in mouse tetraploid←→diploid chimaeras. Dev Biol 167:213–226.
Jing-he T, Qi Z, Li-bin X, Qiu-ming Z. 1994. Factors affecting electrofusion of 2-cell mouseembryos.Theriogenology42:173–181.
Kajii T, Ferrier A, Niikawa N, Takahara H, Ohama K, Avirachan S. 1980. Anatomic and chromosomal anomalies in 639 spontaneous abortuses. Hum Genet 55:87–98.
Kaufman MH. 1991a. Histochemical identification of primordial germ cells and differentiation of the gonads in homozygous tetraploid mouse embryos. J Anat 179:169–181.
Kaufman MH. 1991b. New insights into triploidy and tetraploidy, from an analysis of model systems for these conditions. Hum Reprod 6:8–16.
Kaufman MH. 1992. Postcranial morphological features of homozygous tetraploid mouse embryos. J Anat 180(Pt 3):521–534.
Kaufman MH, McLaren A. 1992. Developmental abnormalities in tetraploid mouse embryos. Ciba Found Symp 165: 108–110.
Kaufman MH, Webb S. 1990. Postimplantation development of tetraploid mouse embryos produced by electrofusion. Development 110:1121–1132.
Kawarsky SJ, Basrur PK, Stubbings RB, Hansen PJ, King WA. 1996. Chromosomal abnormalities in bovine embryos and their influence on development. Biol Reprod 54:53–59.
Koizumi N, Fukuta K. 1995. Preimplantation development of tetraploid mouse embryo produced by cytochalasin B. Exp Anim 44:105–109.
Koizumi N, Fukuta K. 1996. Effect of insulin on in vitro development of tetraploid mouse embryos. Exp Anim 45:179–181.
Keighren M, West JD. 1993. Analysis of cell ploidy in histological sections of mouse tissues by DNA-DNA in situ hybridization with digoxigenin-labelled probes. Histochem J 25:30–44.
Krivokharchenko A, Galat V, Ganten D, Bader M. 2002. In vitro formation of tetraploid rat blastocysts after fusion of two-cell embryos. Mol Reprod Dev 61: 460–465.
Kubiak JZ, Tarkowski AK. 1985. Electrofusion of mouse blastomeres. Exp Cell Res 157:561–566.
Kurischko A, Berg H. 1986. Electrofusion of rat and mouse blastomeres. Bioelectrochem Bioenerg 15:513–519.
Larsen W. 1993. Human embryology. New York: Churchill Livingstone.
Li JX, Ning G, Duncan SA. 2000. Mammalian hepatocyte differentiation requires the transcription factor HNF-4alpha. Genes Dev 14:464–474.
Lo C. 1986. Localization of low abundance DNA sequences in tissue sections by in situ hybridization. J Cell Sci 81:143–162.
Lu TY, Markert CL. 1980. Manufacture of diploid/tetraploid chimeric mice. Proc Natl Acad Sci U S A 77:6012–6016.
Luo J, Sladek R, Bader JA, Matthyssen A, Rossant J, Giguere V. 1997. Placental abnormalities in mouse embryos lacking the orphan nuclear receptor ERR-beta. Nature 388:778–782.
McFeely R. 1969. Aneuploidy, polyploidy, and structural rearrangement of chromosomes in mammals other than man. In: Benirschke K, editor. Comparative mammalian cytogenetics. Hanover, NH: Springer-Verlag. p 434–444.
McGaughey RW, Chang MC. 1969. Inhibition of fertilization and production of heteroploidy in eggs of mice treated with colchicine. J Exp Zool 171:465–480.
McLaughlin K. 1993. Production of tetraploid embryos by electrofusion. In:

DePamphilis M, editor. Methods in enzymology. New York: Academic Press. p 919–29.

Matveeva NM, Shilov AG, Kaftanovskaya EM, Maximovsky LP, Zhelezova AI, Golubitsa AN, Bayborodin SI, Fokina MM, Serov OL. 1998. In vitro and in vivo study of pluripotency in intraspecific hybrid cells obtained by fusion of murine embryonic stem cells with splenocytes. Mol Reprod Dev 50:128–138.

Mishina Y, Crombie R, Bradley A, Behringer RR. 1999. Multiple roles for activin-like kinase-2 signaling during mouse embryogenesis. Dev Biol 213: 314–326.

Misra RP, Bronson SK, Xiao Q, Garrison W, Li J, Zhao R, Duncan SA. 2001. Generation of single-copy transgenic mouse embryos directly from ES cells by tetraploid embryo complementation. BMC Biotechnol 1:12.

Modlinski JA. 1978. Transfer of embryonic nuclei to fertilised mouse eggs and development of tetraploid blastocysts. Nature 273:466–467.

Modlinski JA. 1981. The fate of inner cell mass and trophectoderm nuclei transplanted to fertilized mouse eggs. Nature 292:342–343.

Nagy A, Rossant J. 1993. Production of completely ES cell-derived fetuses. In: Joyner A, editor. Gene targeting: a practical approach. Oxford: IRL Press.

Nagy A, Rossant J. 2001. Chimaeras and mosaics for dissecting complex mutant phenotypes. Int J Dev Biol 45:577–582.

Nagy A, Gocza E, Diaz EM, Prideaux VR, Ivanyi E, Markkula M, Rossant J. 1990. Embryonic stem cells alone are able to support fetal development in the mouse. Development 110:815–921.

Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC. 1993. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc Natl Acad Sci U S A 90:8424–8428.

Niemierko A, Opas J. 1978. Manipulation of ploidy in the mouse. In: Daniel J Jr, editor. Methods in mammalian reproduction. New York: Academic Press. p 49–65.

O'Neill GT, Speirs S, Kaufman MH. 1990. Sex-chromosome constitution of postimplantation tetraploid mouse embryos. Cytogenet Cell Genet 53:191–195.

Osborn TC, Pires JC, Birchler JA, Auger DL, Chen ZJ, Lee HS, Comai L, Madlung A, Doerge RW, Colot V, Martienssen RA. 2003. Understanding mechanisms of novel gene expression in polyploids. Trends Genet 19:141–147.

Ozil JP, Modlinski JA. 1986. Effects of electric field on fusion rate and survival of 2-cell rabbit embryos. J Embryol Exp Morphol 96:211–228.

Peli J, Schmoll F, Laurincik J, Brem G, Schellander K. 1996. Comparison of aggregation and injection techniques in producing chimeras with embryonic stem cells in mice. Theriogenology 45: 833–842.

Perry MM, Snow MH. 1975. The blebbing response of 2-4 cell stage mouse embryos to cytochalasin B. Dev Biol 45:372–377.

Petzoldt U. 1991. Developmental profile of glucose phosphate isomerase allozymes in parthenogenetic and tetraploid mouse embryos. Development 112:471–476.

Pincus G, Waddington C. 1939. The effects of mitosis inhibiting treatments on normally fertilized pre-cleavage rabbit eggs. J Hered 30:515–518.

Prather RS, Hoffman KE, Schoenbeck RA, Stumpf TT, Li J. 1996. Characterization of DNA synthesis during the 2-cell stage and the production of tetraploid chimeric pig embryos. Mol Reprod Dev 45:38–42.

Rideout WM, Hochedlinger K, Kyba M, Daley GQ, Jaenisch R. 2002. Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. Cell 109:17–27.

Rieger R, Michaelis A, Green M. 1991. Glossary of genetics: classical and molecular. Berlin: Springer-Verlag.

Rossant J, Guillemot F, Tanaka M, Latham K, Gertenstein M, Nagy A. 1998. Mash2 is expressed in oogenesis and preimplantation development but is not required for blastocyst formation. Mech Dev 73:183–191.

Schmutz SM, Lin CC. 1983. Enzyme expression in tetraploid human fibroblasts. Am J Hum Genet 35:53A.

Schorpp-Kistner M, Wang ZQ, Angel P, Wagner EF. 1999. JunB is essential for mammalian placentation. EMBO J 18: 934–948.

Schreiber M, Wang ZQ, Jochum W, Fetka I, Elliott C, Wagner EF. 2000. Placental vascularisation requires the AP-1 component fra1. Development 127:4937–4948.

Sekirina GG, Bogolliubova NA, Antonova NV, Dyban AP. 1997. The behaviour of mitochondria and cell integration during somatic hybridisation of sister blastomeres of the 2-cell mouse embryo. Zygote 5:97–103.

Senda M, Takeda J, Abe S, Nakamura T. 1979. Induction of cell fusion of plant protoplasts by electric stimulation. Plant Cell Physiol 20:1441–1443.

Shawlot W, Wakamiya M, Kwan KM, Kania A, Jessell TM, Behringer RR. 1999. Lim1 is required in both primitive streak-derived tissues and visceral endoderm for head formation in the mouse. Development 126:4925–4932.

Sheppard DM, Fisher RA, Lawler SD, Povey S. 1982. Tetraploid conceptus with three paternal contributions. Hum Genet 62:371–374.

Sibilia M, Steinbach JP, Stingl L, Aguzzi A, Wagner EF. 1998. A strain-independent postnatal neurodegeneration in mice lacking the EGF receptor. EMBO J 17: 719–731.

Sirard C, de la Pompa JL, Elia A, Itie A, Mirtsos C, Cheung A, Hahn S, Wakeham A, Schwartz L, Kern SE, Rossant J, Mak TW. 1998. The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later for anterior development of the mouse embryo. Genes Dev 12:107–119.

Smith R, McLaren A. 1977. Factors affecting the time of formation of the mouse blastocoele. J Embryol Exp Morphol 41: 79–92.

Snow MH. 1973. Tetraploid mouse embryos produced by cytochalasin B during cleavage. Nature 244:513–515.

Snow MH. 1975. Embryonic development of tetraploid mice during the second half of gestation. J Embryol Exp Morphol 34:707–721.

Snow MH. 1976. The immediate postimplantation development of tetraploid mouse blastocysts. J Embryol Exp Morphol 35:81–86.

Spindle A. 1981. Polyethylene glycol-induced fusion of two-cell mouse embryo blastomeres. Exp Cell Res 131:465–470.

Spindle A, Sturm KS, Flannery M, Meneses JJ, Wu K, Pedersen RA. 1996. Defective chorioallantoic fusion in mid-gestation lethality of parthenogenone⟷tetraploid chimeras. Dev Biol 173:447–458.

Surti U, Szulman AE, Wagner K, Leppert M, O'Brien SJ. 1986. Tetraploid partial hydatidiform moles: two cases with a triple paternal contribution and a 92,XXXY karyotype. Hum Genet 72:15–21.

Tallquist MD, Soriano P. 2000. Epiblast-restricted Cre expression in MORE mice: a tool to distinguish embryonic vs. extra-embryonic gene function. Genesis 26:113–115.

Tan J, Zhou Q, Xu L, Zhang Q, Qin P. 1994. Factors affecting electrofusion of 2-cell mouseembryos.Theriogenology42:173–181.

Tanaka M, Gertsenstein M, Rossant J, Nagy A. 1997. Mash2 acts cell autonomously in mouse spongiotrophoblast development. Dev Biol 190:55–65.

Tang PC, West JD. 2000. The effects of embryo stage and cell number on the composition of mouse aggregation chimeras. Zygote 8;235–243.

Tarkowski AK. 1972. Comments on the application of cell fusion techniques to the study of mammalian embryology. Adv Biosci 8:187–202.

Tarkowski AK, Witkowska A, Opas J. 1977. Development of cytochalasin B-induced tetraploid and diploid/tetraploid mosaic mouse embryos. J Embryol Exp Morphol 41:47–64.

Tarkowski AK, Ozdzenski W, Czolowska R. 2001. How many blastomeres of the 4-cell embryo contribute cells to the mouse body? Int J Dev Biol 45:811–816.

Terada N, Hamazaki T, Oka M, Hoki M, Mastalerz DM, Nakano Y, Meyer EM, Morel L, Peterson BE, Scott EW. 2002. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 416:542–545.

Vogel R, Spielmann H. 1987. Spontaneous and cyclophosphamide-induced sister-chromatid exchanges in diploid and endoreduplicated tetraploid met-

aphases of preimplantation mouse embryos. Mutat Res 192:137–140.

Voss AK, Thomas T, Gruss P. 2000. Mice lacking HSP90beta fail to develop a placental labyrinth. Development 127: 1–11.

Wang ZQ, Kiefer F, Urbanek P, Wagner EF. 1997. Generation of completely embryonic stem cell-derived mutant mice using tetraploid blastocyst injection. Mech Dev 62:137–145.

Warburton D, Byrne J, Canki N. 1991. Chromosome anomalies and prenatal development: an atlas. New York: Oxford University Press.

Webb S, de Vries TJ, Kaufman MH. 1992. The differential staining pattern of the X chromosome in the embryonic and extraembryonic tissues of postimplantation homozygous tetraploid mouse embryos. Genet Res 59:205–214.

Wood SA, Allen ND, Rossant J, Auerbach A, Nagy A. 1993. Non-injection methods for the production of embryonic stemcell-embryochimaeras.Nature365: 87–89.

Yamamoto H, Flannery ML, Kupriyanov S, Pearce J, McKercher SR, Henkel GW, Maki RA, Werb Z, Oshima RG. 1998. Defective trophoblast function in mice with a targeted mutation of Ets2. Genes Dev 12:1315–1326.

Ying Q, Nichols J, Evans EP, Smith AG. 2002. Changing potency by spontaneous fusion. Nature 416:545–548.